PROTEIN, CALORIE AND METHIONINE INTAKES IN RELATION TO NITROGEN BALANCE

(A NOTE ON THE PAPER BY MERTZ ET AL.)

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A recent study by Mertz et al. ('52) is concerned with the intake of essential amino acids on self-selected diets. The latter authors conclude that "Methionine appears to be the first limiting essential amino acid in the self-selected diets studied" and that the diets could probably be "improved by the addition of foods rich in methionine." This conclusion was obtained by comparing the intake of the various amino acids with the strictly "tentative standards" for amino acid intakes proposed by Rose ('49) for young adult males. Many of the subjects were consuming less protein than the amounts recommended by the Food and Nutrition Board of the National Research Council ('48) and many were in negative nitrogen balance.

It is well known that nitrogen balance may be influenced not only by the total nitrogen and amino acid intake, but by previous dietary history, calorie intake, body size, perhaps age, and so forth. In view of the large number of possible important variables, some of which are interrelated, a simple inspection of such data may lead to erroneous conclusions. It is possible to evaluate the relative importance of the several variables as they effect nitrogen balance by calculation of the partial correlation coefficients (Yule and Kendall, '37). This has been done for the 4 variables: nitrogen balance (1),

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nitrogen intake (2), calorie intake (3) and methionine intake (4), using the data presented by Mertz et al. ('52). The results are presented in table 1.

TABLE 1

Total and partial correlation coefficients for the 4 variables, nitrogen balance (1), nitrogen intake (2), calorie intake (3), and methionine intake (4)

CORRELATION COEFFICIENT (zero order)[1]		CORRELATION COEFFICIENT (second order)[2]	
r_{12}	0.820	$r_{12.34}$	0.777
r_{13}	0.437	$r_{13.24}$	0.342
r_{14}	0.485	$r_{14.23}$	— 0.211
r_{23}	0.657	$r_{23.14}$	0.316
r_{24}	0.626	$r_{24.13}$	0.477
r_{34}	0.676	$r_{34.12}$	— 0.145

[1] These are the correlations found from the raw data. The value for r_{12} represents the coefficient of correlation between nitrogen balance and nitrogen intake; r_{13} between nitrogen balance and calorie intake, etc.

[2] The value for $r_{12.34}$ represents the correlation between nitrogen balance and nitrogen intake when the remaining variables, calorie intake and methionine intake, are kept constant.

Considering the total or zero order correlation coefficients first, it will be observed that the correlation of nitrogen balance with nitrogen intake, r_{12}, is considerably larger than any of the others, whereas the correlations of nitrogen balance with calorie intake and with methionine intake are the smallest values obtained. The latter value was obtained in spite of a higher correlation between protein and methionine intakes, r_{24}. *All* of the coefficients are statistically significant at the 5% level of probability, and all except r_{13} at the 1% level.

The partial correlation coefficients are much more instructive. The correlation of nitrogen intake with balance, independent of the effects of calorie and methionine intake, $r_{12.34}$, is the only coefficient which is statistically significant at either the 5 or 1% level of probability. Although it is not significant statistically, it is of some interest that the partial correlation between nitrogen balance and methionine

intake, $r_{14.23}$, is actually a negative one. As might be expected, the correlation of protein intake and methionine intake, $r_{24.13}$, approaches significance, since the protein intake includes the methionine intake.

It may be concluded that the paper under discussion provides no evidence that methionine is a limiting amino acid in the diets studied. Calorie intakes also appear relatively unimportant in this group as a whole and the total nitrogen intake is by far the most important factor in determining nitrogen balance. The same type of analysis might be extended to determine the possible importance of body size, age, and so forth. However, in view of the dominant part played by the protein intake and the limited data available, this does not appear to be worthwhile.

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EDITORIAL ADDENDUM

Dr. Hegsted kindly consented to having his paper sent to Doctors Mertz and associates for comment. A companion study by Futrell et al. (J. Nutrition, *46:* 299, March, 1952) also reported methionine to be a "limiting" amino acid in certain diets that were investigated. These authors, likewise, were given an opportunity to see the Hegsted manuscript and to comment on it. The replies of these two groups of authors are given below.

Comments by Mertz and associates

It is unfortunate that Dr. Hegsted has not been able to contribute data from his own laboratory on this subject. We fear that our data, which we term "a preliminary survey" which has "helped to point up several areas in which research needs to be done" (p. 320, Mertz et al., '52), are being overworked. We therefore urge the

reader to examine the original paper by Mertz et al. before accepting Dr. Hegsted's conclusions.

Using our data on 18 subjects, Dr. Hegsted has calculated correlation coefficients relating nitrogen and calorie intakes to nitrogen balance. We considered the advisability of including this analysis, but decided that it would be better if these limited data were included in the statistical analysis of the balance data from the entire group of 136 subjects studied in the North Central Regional cooperative experiments. The reader is therefore referred to the paper entitled "Intakes and Retentions of Nitrogen, Calcium and Phosphorus by 136 Women Between 30 and 85 Years of Age" by M. A. Ohlson et al. (Fed. Proc., *10:* 775, September, 1952). We believe that the correlation of nitrogen and calorie intakes with nitrogen balance in the paper cited is more significant than that made by Dr. Hegsted on 18 of the 136 subjects.

It should be emphasized that no fault was found with the analytical methods we used, or the accuracy of the data. Dr. Hegsted disagrees with our interpretation of the results. By calculation of partial correlation coefficients, using our data, he has found that no correlation exists between methionine intake and nitrogen balance. Therefore, to quote Dr. Hegsted, "the paper under discussion provides no evidence that methionine is a limiting amino acid in the diets studied."

In the paper by Mertz et al. no attempt is made to relate methionine intake to nitrogen balance. If all of the subjects in table 2 (p. 318) were in *positive* nitrogen balance and the amino acid intakes were those shown in this table, we would still be justified in concluding that, *based on Rose's minimum intakes,* methionine is the first limiting amino acid in these self-selected diets. We determined the daily intake pattern of essential amino acids consumed by our subjects and compared this with the intake pattern of essential amino acids found by Rose to be required in controlled experiments on human adult males.

We do not agree with Dr. Hegsted's statement that "It is possible to evaluate the relative importance of the several variables as they affect nitrogen balance by calculation of the partial correlation coefficients." Only 4 variables — nitrogen balance, protein intake, calorie intake and methionine intake — have been included in the analyses. How much significance can be attached to these partial correlation coefficients when there are variables for which Dr. Hegsted has no data? Thus, data are lacking on the effect of the following variables on nitrogen balance: (1) Distribution of protein in self-selected meals throughout the day, (2) methionine-sparing action of the cystine in the self-selected diets, (3) change in methionine requirement with change in level (and source) of calories fed, (4) level in these self-

selected diets of vitamin B_{12}, pyridoxine and other vitamins associated with metabolism of amino acids, and (5) previous dietary history, body size, age, emotional state of subject and sex. These 11 or more variables should be included and partial correlations calculated. Unless this is done, we *cannot know* the relation of methionine and nitrogen balance *"independent of the other variables."*

Because of the uncertainties introduced by lack of data on the above variables, a statistical analysis of the methionine data in table 2 (p. 318) of the paper by Mertz et al. appears to us to be unwarranted and perhaps even misleading. Therefore, we compared our amino acid data with the one set of "constants" available at present; namely, the minimum intakes determined by Dr. Rose. We believe that only a simple comparison of this nature is justified.

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Comments by Futrell and associates

In a companion study (J. Nutrition, *46:* 299, 1952) to that of Mertz et al., we also observed that the amounts of methionine in a series of self-selected diets were "limiting" with respect to the tentative standards proposed by Rose (Fed. Proc., *8:* 546, 1949), although the amounts of all of the other essential amino acids in the diets were above the recommended levels. It was pointed out that Rose's recommendation for methionine was based on the responses of subjects who were not receiving any cystine, and that this recommendation is probably too high when applied to cystine-containing natural diets. The question is, therefore, still open as to whether any substantial number of average adults on self-selected diets are in negative nitrogen balance because of an insufficient intake of methionine (the sense in which the word "limiting" is used by Dr. Hegsted), although the possibility may exist in certain individuals. Preliminary attempts to improve an obviously poor diet by supplementation with methionine have thus far failed to improve N retention (M. F. Futrell, Ph.D. thesis, University of Wisconsin, 1952).

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THE FEED VALUE OF FRESH BONE

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The problem of providing adequate nutrition for large numbers of people has revived interest in the use of fresh bone as a source of minerals and possibly as a protein supplement for foods. In France, about 150 years ago, the preparation of gelatin from bone by pressure cooking and its use as a substitute for meat aroused the controversy over the food value of gelatin which was finally settled by Magendie (1841). Carl Voit (1874) conducted several balance trials using ossein, the organic material of bone which remained after cooking the bone with hydrochloric acid. This preparation decreased the nitrogen loss of starvation but would not completely eliminate the negative nitrogen balance.

From that time until World War II there was little attention paid to bone as a food material. Following the war, the interest in bone as a source of calcium and phosphorus for emergency food supplies was revived. Drake et al. ('49) made studies on foods containing fresh bone prepared for use in Europe and, using balance trials on human subjects, compared the availability of bone calcium with that of milk and found them nearly equal under the conditions of their experiment.

PROCEDURE

The bone used for this study was obtained from the beef boning operations of a local meat packing plant, in two lots, one of 500 lb. and the other of 300 lb., and was a mixture of all the

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carcass bones except the skull, cannon bones and feet. The bones were put through a large bone grinder [2] which ground them into a rather coarse particle size, ranging up to one-eighth to one-fourth inch. This coarsely ground bone was packed in large cartons and kept frozen until used. When it appeared desirable to use a smaller particle size, the frozen bone was mixed with dry ice and ground through a micro-pulverizer [3] or mixed in the diet and then pulverized.

Purebred, immature beagles were used on balance trials to determine the availability of the calcium and phosphorus.

TABLE 1

Composition of bone

CONSTITUENT	LOT 1 (Of dry matter)	LOT 2 (Of dry matter)
Crude protein (N × 6.25)	28.1%	28.8%
Nitrogen	4.5%	4.6%
Ether extract	32.2%	23.6%
Total ash	36.7%	43.8%
Calcium	140.6 mg/gm	
Phosphorus	61.1 mg/gm	
Magnesium	4.2 mg/gm	

Adult purebred beagles were also used on balance trials to determine the availability of the crude protein of the bone and to determine the effects of several treatments on the availability of this protein. For the studies on the effect of amino acid supplementation of bone diets, immature white rats were used. The compositions of the two lots of bone used are given in table 1.

The quantity of diet required for each balance trial and for the rat trials was prepared in advance and kept frozen until used. The urine samples were preserved with hydrochloric acid and toluene. The feces were collected daily and kept frozen until prepared for analysis. The collections from

[2] Manufactured by the F. W. Mann Co., Milford, Massachusetts.

[3] Manufactured by the Pulverizing Machinery Corp., Summit, New Jersey.

the calcium balance trials in which pulverized bone was used were made into slurries, using a Waring Blendor, and sampled by weight. They were then digested by preliminary treatment with concentrated nitric acid followed by perchloric acid. In the nitrogen balance trials where coarse bone was used, the fecal samples were dried in a Desivac, ground in a Wiley mill and sampled.

Calcium was determined by precipitation as the oxalate and titration with ceric ammonium nitrate in perchloric acid at room temperature, as described by Smith ('42). Phosphorus was determined colorimetrically by the phosphovanadomolybdate method of Simonsen et al. ('46). Nitrogen was determined by the macro Kjeldahl method, and the amino acid determination of the bone protein was done by the microbiological technique.

RESULTS

I. Availability of the calcium and phosphorus of bone

Two litters of beagles were used for these trials. Group 1 was a litter of 6, group 2 a litter of 5. The trials were started when the animals were about 80 days of age and continued to 200 days of age for group 1 and 330 days for group 2. A 5-day adjustment period, followed by a 7-day collection period, was used for each balance. The dogs were exercised regularly between collection periods and were maintained on stock dog feeds between trials.

The percentage composition of the diet used in these trials was as follows: corn flakes, 55; dried yeast, 8; lard, 10; cod liver oil, 2; horse meat (dry weight basis), 20; mineral mix, 2; and bone 3. The bone used had been ground through the micropulverizer. No meat was available when diet 14 was prepared, and more corn flakes and lard were used in place of it. The mineral mix for group 1 was the low calcium mixture of Adolph et al. ('38). The mixture for group 2 was prepared to supply the required minerals other than calcium, phosphorus and magnesium.

The analyses for calcium and phosphorus of the diets used in trials 6 through 14 are given in table 2. Phosphorus was not determined for the diets for group 1.

TABLE 2

Calcium and phosphorus contents of diets and of dry matter in diets

DIET	CALCIUM	PHOSPHORUS
	%	%
6	0.401	
7	0.296	
8	0.333	
9	0.342	
10 and 11	0.433	0.466
12	0.441	0.476
13	0.494	0.549
14	0.452	0.543

TABLE 3

Results of the calcium balance trials (group 1)

Trial number	6	7	8	9
Age in days	80	110	160	200
Average weight (kg)	2.1	2.9	6.2	7.0
Intake (mg/kg/day)	305	168	138	120
Excretion				
Feces (mg/kg/day)	134	70	90	88
Urine (mg/kg/day)	3	5	1	1
Absorption (mg/kg/day)	171	98	48	32
Absorbed (%)	56	58	35	26
Retention (mg/kg/day)	168	93	47	31
Retained (%)	55	55	34	26
Total absorbed (mg/day)	360	284	298	224

The results with group 1 (6 beagles) are given in table 3. The results for the calcium and phosphorus balances for group 2 (5 beagles) are given in table 4.

In group 1 56 to 58% of the calcium of bone was absorbed by immature beagles of 80 to 110 days of age at a level of intake of 0.4% of the dry weight of the diet. The calcium requirement of the dog can be satisfied with fresh, untreated bone meal when it is included in the diet at this level. The

TABLE 4

Calcium and phosphorus balances (group 2)

CATEGORY OF INTEREST	NUMBER OF THE BALANCE TRIAL									
	10		11		12		13		14	
	Ca	P	Ca	P	Ca	P	Ca	P	Ca	P
No. of dogs	5	5	5	5	5	5	5	5	5	5
Age (days)	80		120		160		200		330	
Average weight (kg)	3.5		5.2		6.4		7.0		8.9	
Intake (mg/kg/day)	184	198	154	166	135	146	155	172	123	147
Excretion (mg/kg/day)										
Feces	91	101	119	114	112	96	125	107	103	80
Urine	3	52	3	37	2	71	2	34	4	53
Absorption (mg/kg/day)	93	97	35	52	23	50	30	65	20	67
Absorbed (%)	51	49	23	31	17	34	19	38	16	45
Retention (mg/kg/day)	90	45	32	15	21	—21	28	31	16	14
Retained (%)	49	23	21	9	16	0	18	18	13	10
Total absorbed (mg/day)	330	342	178	270	147	320	210	455	178	605

total amount absorbed per day, disregarding body weight, decreased slowly as the dog matured and the rate of skeletal growth decreased. In group 2, 51% of the calcium and 49% of the phosphorus in the diet were absorbed at 80 days of age. As the dogs grew older, the total daily absorption of phosphorus without regard to body weight tended to increase after 120 days of age, as opposed to the daily total calcium absorption, which tended to remain constant.

II. Availability of the protein

Purebred, adult beagles were used for these 4 trials. The technique was similar to that used for the mineral balances. The fecal collections were kept frozen until dried for analysis in a Desivac. All nitrogen analyses were done by the macro Kjeldahl procedure.

The percentage composition of the diets was as follows: fresh bone, 30; cooked cornstarch, 65; cod liver oil, 2; yeast, 1.5; and sodium chloride, 1.5. For balance 15 the diet was prepared using the coarsely ground bone, and for balance 16 the diet was prepared in a similar way but the entire diet was reground through the micropulverizer to determine whether particle size had any effect. The coarse bone used for trial 17 was pressure cooked in the autoclave at 15 lb. pressure for 4 hours before being mixed into the diet. For trial 18 an equal weight of 2% hydrochloric acid was mixed with the bone and then autoclaved at 12 to 15 lb. pressure for 4 hours, and the diet mixed. These treatments were used to determine whether a partial destruction of the bone structure would improve the availability and quality of the protein for the animal.

In all 4 trials, the dogs were in negative nitrogen balance, due to the deficiency of methionine in the protein structure. For these studies the alleviation of the nitrogen loss was used as a measure of the effect of the treatment of the bone. The results of these 4 balances are presented in table 5; the values in this table are for nitrogen expressed as milligrams per kilogram of body weight per day.

The apparent absorptions in these trials were similar except for that in trial 16, which was low; only 93 mg/kg/day were absorbed. This may have been due to the bulk of finely divided mineral matter in this diet which resulted in a mechanical obstruction and inefficient absorption of the protein. The per cent absorptions for the other three balances were the same and are the best estimate of the availability of the crude nitrogen of the bone.

A statistical comparison of these results was made with the binomial probability paper of Mosteller and Tukey ('49) by plotting the nitrogen intake in milligrams/kilogram/day against the nitrogen balance in milligrams/kilogram/day.

TABLE 5

Results of the nitrogen balance trials 15 through 18

Trial number	15	16	17	18
No. of dogs	6	5	5	6
Intake (mg/kg/day)	299	316	325	308
Excretion (mg/kg/day)				
Feces	160	223	179	169
Urine	253	217	235	175
Absorption (mg/kg/day)	139	93	147	139
Absorbed (%)	46	30	46	45
Balance (mg/kg/day)	−114	−124	−89	−36

This comparison indicated that the pulverizing of the diet in trial 16 had no effect upon the nitrogen balance. The nitrogen balance data for the diet of trial 17 were between the 5% and 1% levels of significance when compared with the nitrogen balance data of trials 15 and 16; hence this difference was probably due to the treatment. The nitrogen balance data of trial 18 were highly significant when compared with those of trials 15 and 16 or trial 17, and the difference can be ascribed to the treatment. These differences suggest that the pressure cooking and the use of 2% hydrochloric acid altered the structure of the bone in such a way as to improve the timing of the absorption of the amino acids from the digestive tract, thus improving the utilization and

decreasing the nitrogen loss. This is borne out by the decrease in urinary nitrogen excreted in trial 18.

III. Amino acid supplementation of bone protein

The following studies on the supplementation of bone protein with pure amino acids were done with immature white rats of 30 to 40 gm in body weight. Several preliminary trials with small groups of rats were run to determine roughly which amino acids were likely to be most effective, since the

TABLE 6

Rat trial 3, weight gain and feed consumption

DIET	DESCRIPTION	NO. OF RATS	AVE. TOTAL GAIN FOR GROUP	AVE. TOTAL FEED CONSUMED	AVE. GAIN PER GM FEED
			gm	*gm*	*gm*
1	Positive control, 10 essential amino acids	10	93.4	314	0.297
2	Negative control	10	76.7	356	0.215
3	0.6% Methionine	10	91.9	326	0.282
4	0.6% Methionine plus 0.2% tryptophan	10	91.6	328	0.279
5	0.6% Methionine plus 0.4% histidine	10	82.5	297	0.278
6	0.6% Methionine plus 0.4% isoleucine	10	90.8	325	0.279

amino acid composition data for gelatin indicated a multiple deficiency (Rose, '36). In this trial the growth response per gram of feed consumed was used as the criterion of the effect of supplementation. The basic diet was composed of fresh bone, 50; cod liver oil, 2; sodium chloride, 1; and a composite vitamin B supplement in sugar, 1 part; and was designed to supply all the B complex requirements of the rat. The level of cooked cornstarch was adjusted for each of the 5 amino acid mixtures added and the negative control diet to give a total of 100 parts. Diet 1 was the basic ration supplemented

with all 10 essential amino acids at the levels suggested by Rose ('37); diet 2 contained no supplement to the basal diet; diet 3 contained 0.6% DL-methionine; diet 4, 0.6% DL-methionine and 0.2% DL-tryptophan; diet 5, 0.6% DL-methionine and 0.4% L-histidine monohydrochloride; and diet 6, 0.6% DL-methionine and 0.4% DL-isoleucine. Ten rats were used on each diet for a total of 27 days. The growth results are given in table 6.

The group receiving the complete supplement of all 10 amino acids gained 0.297 gm for each gram of feed consumed. The methionine-supplemented groups gained 0.282 gm/gm of feed, nearly equal to the positive controls, and the addition of tryptophan, histidine, or isoleucine gave no improvement over methionine alone. The negative control group gained 0.215 gm/gm of feed, the least of any group even though they consumed the most feed. As determined by binomial probability paper, the rate of gain of the negative control group was significantly different than that of the other 5 groups at the 5% level. There was no significant difference among the other groups. These data indicate that for practical supplementation, methionine is the limiting amino acid in crude bone protein.

A microbiological determination of the amino acid composition was also done on a sample of the fresh ground bone. The protein was hydrolyzed by acid and the results are presented in table 7 in terms of grams of amino acid per 16 gm of nitrogen. The values for the amino acid content of the bone protein obtained from the microbiological assay were used to calculate the amino acid content per 100 gm of the negative control diet. In column three of table 7 are given the accepted requirements per 100 gm of diet as given by Rose ('37). In column 4 are the ratios of the amounts calculated to be in negative control diet no. 2 to the accepted values of Rose. From this calculation it can be seen that there is a high proportion of arginine in bone protein, which probably accounts for the higher requirement of this amino acid in the diet of the growing animal than in the adult. This method

of calculation also emphasizes the deficiency of this protein in the amino acid, methionine. The remaining amino acids are found to be present in about the accepted relative amounts but in about half the absolute quantities suggested by Rose. Brinegar et al. ('50), in studies on the amino acid requirements of swine, emphasized the importance of considering the level of dietary protein in evaluating the individual amino acid requirements. The presence of adequate amounts of choline

TABLE 7

Amino acid composition of bone protein and basal rat diet

AMINO ACIDS	GM AMINO ACID PER 16 GM NITROGEN	CALCULATED GM AMINO ACID PER 100 GM OF DIET 2 (TABLE 6)	ACCEPTED [1] REQUIREMENT, GM AMINO ACIDS PER 100 GM OF DIET	RATIO OF ACCEPTED REQUIREMENT TO CALCULATED CONTENT IN BASAL RAT DIET
Arginine	8.1	1.0	0.2	5.0
Histidine	1.1	0.15	0.4	0.37
Isoleucine	2.7	0.30	0.5	0.60
Leucine	3.5	0.40	0.9	0.45
Lysine	4.7	0.60	1.0	0.60
Methionine	0.7	0.10	0.6	0.15
Phenylalanine	2.3	0.30	0.7	0.45
Threonine	2.5	0.30	0.6	0.50
Valine	3.0	0.40	0.7	0.55

[1] Rose ('37).

in the vitamin mixture probably accounts for the growth of the negative control group in the presence of the apparent deficiency of methionine.

DISCUSSION

The desirability of finding and using good, cheap mineral supplements for feeds is increasing at the present time, and the use of ground fresh bone meal in some dog feeds has been adopted. A potentially large source of calcium and phosphorus exists in the bone by-products of the meat packing industry, very little of which finds its way, in the fresh state, into feeds. Whole fresh bone poses a storage problem but can be kept well when frozen.

It is apparent from the mineral balances reported that fresh bone can be used to satisfy the mineral requirements of the young growing dog. While the 56% availability found is not as high as the 70 to 80% reported for the calcium of milk by McCay ('49), there are many factors which influence the absorption of calcium, such as the concentration of calcium in the diet, the solubility of the salt, the acidity of the digestive tract, and the presence of carbohydrates and phytate.

By calculation, about 40% of the phosphorus in these mineral balances was supplied from the bone and the remaining 60% came from the meat and other ingredients of the diet.

The extensive use of bone as a source of protein is not practical, but it is of interest that the quality of crude bone protein appears to be better than that of the steam-extracted protein, gelatin, which is notoriously poor as a protein. This is compatible with the fact that steam extraction removes only about 75% of the protein from bone. The improvement in the quality with acid treatment and pressure cooking is apparently due to the hydrolysis and the increased availability of the amino acid with better timing of absorption from the digestive tract.

SUMMARY

1. From a series of mineral balances using two litters of growing beagles, the availability of the calcium of ground, fresh, whole bone was found to be 56%. The availability of the phosphorus in these diets, 40% of which was supplied by the bone, was 49%.

2. From nitrogen balances on adult beagles fed diets in which the protein was supplied by fresh bone, it was found that 45% of the protein was available to the dog.

3. Treatment of the bone with acid and pressure cooking decreased the nitrogen loss as compared with untreated bone, whether coarsely or finely ground.

4. A microbiological determination of the amino acid composition of bone protein showed methionine to be the limiting amino acid.

5. Supplementation with methionine of a diet in which bone supplied the protein produced good growth in young rats, and further supplementation with histidine or isoleucine did not give any added growth.

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FACTORS AFFECTING THE STABILITY OF THIAMINE IN A TYPICAL LABORATORY DIET[1]

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ONE FIGURE

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The lability of vitamin B_1 to heat, alkali or bisulfite has long been known, but the degree to which the vitamin might be unstable in ordinary laboratory diets has not been emphasized. Recently, however, growth failure and neurological symptoms were observed in rats fed certain low-protein, niacin-free diets, and these symptoms were cured by the injection of thiamine (Hankes and Elvehjem, '49). Lyman and Elvehjem ('51) demonstrated that 80% of the thiamine in synthetic rations may disappear after two weeks at room temperature, while succinylsulfathiazole tends to preserve the vitamin. In 1951, rats and chicks in the students' animal laboratory of this Department failed to grow properly on a series of synthetic diets in which the original level of thiamine was adequate, and growth was restored by thiamine supplements. Other unpublished examples of accidental thiamine deficiency on purified diets have since come to our attention. The present study represents an attempt to reduce to a minimum the destruction of thiamine in laboratory diets.

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METHODS

The basal ration was similar to those used in many current nutritional studies. It had the following percentage composition: sucrose, 71; casein, 20; salts (Wesson, '32), 4; corn oil, 5 (containing 0.02% halibut liver oil and 0.003% α-tocopherol); and the following vitamins, in milligrams per kilogram of diet: riboflavin, 3.0; pyridoxine, 2.0; nicotinic acid, 25.0; calcium pantothenate, 20.0; 2-methyl 1,4-naphthoquinone, 10.0; biotin, 0.1; folic acid, 2.0; choline, 1,000.0; vitamin B_{12}, 0.02, thiamine 0.5 to 20.0. Thiamine was added as a separate 0.05% mixture with sucrose. The rations were mixed in quantities of 250 or 500 gm and kept in brown-glass reagent bottles. Part of each diet was stored in the refrigerator and part at room temperature. Certain diets were also stored in an incubator at 37°C. Variations in the rations included the addition of ascorbic acid, glycerol or antibiotics or the alteration or omission of fat or salts; certain diets were dehydrated or were stored under nitrogen (Kandutsch, '52). The diets were analyzed for thiamine by the thiochrome method as recommended by the Association of Vitamin Chemists ('51), except that the enzymatic digestion step was omitted. Refrigerated samples were analyzed within one to two days after mixing, and all diets were analyzed at intervals thereafter. When vitamin C was determined, the dichlorophenolindophenol titration method was used.

EXPERIMENTAL

Effect of temperature and concentration

As in experiments by others, thiamine was found to be unstable in the basal ration, the losses at room temperature averaging about 70% in one week (table 1, diets 1, 5, 13, 20, 24, 28). The irregularities in these values were attributed to fluctuations in the temperature of the room, thiamine being relatively stable at 4°C., the temperature of the refrigerator, in which losses of thiamine seldom reached 50% in 4 weeks (table 1). Figure 1 illustrates the evenness of the

TABLE 1

Stability of thiamine in various laboratory rations

RATION NO.	CHANGES IN BASAL RATION [1]	PER CENT THIAMINE [2] REMAINING				
		Refriz.		Room temp.		
		1–2 days	28 days	6–7 days	14 days	21–22 days
		%	%	%	%	%
1	None	103	48	21	14	4
2	20 mg B_1/kg	106	51	25	13	4
3	No fat, no salts	103	99	76	70	58
4	5 gm ascorbic acid/kg	98	91	89	93	92
5	None	97	56	32		9 [3]
6	500 mg ascorbic acid/kg	97	91	78		62 [3]
7	No fat	100	90	46		19 [3]
8	No fat + 500 mg ascorbic acid/kg	107	88	72		28 [3]
9	1% corn oil, 5% Crisco	102	78	73		13 [3]
10	1% corn oil + 500 mg ascorbic acid/kg	99	87	92		55 [3]
11	0.009% α-tocopherol	100	44	27		9 [3]
12	0.009% α-tocopherol + 500 mg ascorbic acid/kg	95	87	80		47 [3]
13	None	90	76	68	36	30
14	500 mg ascorbic acid/kg	88	85	95	86	69
15	5% linseed oil	98	55	53	20	6
16	5% linseed oil + 500 mg ascorbic acid/kg	99	77	72	63	45
17	Oxidized salts	99	61	32	16	6
18	No salts	107	104	97	86	45
19	No salts + 500 mg ascorbic acid/kg	110	107	113	97	71
20	None	91		22	15	
21	No salts	102		85	23	
22	25 mg penicillin/kg	91		22	15	
23	25 mg aureomycin/kg	92		22	15	
24	None	92		28	11	
25	Dehydrated *in vacuo*	97		101	100	
26	Exposed to light	92		25	11	
27	In dark	92		25	11	
28	None	101		20	6	
29	Dehydrated in air	98		69	50	
30	Under N_2	101		49	30	
31	12.8% dry kidney	82		89	98	
32	1.2% yeast	89		115	96	
33	2% glycerol	100		104	100	
34	1% glycerol, no fat	93		91	88	

[1] In all cases, the diets directly comparable are those that follow the group labelled "none"; namely, groups 1–4, 5–12, 13–19, etc.

[2] Initial thiamine concentration in all diets except diet 2 was 2 mg/kg.

[3] Assay values for diets 5–12 were obtained on the 19th day.

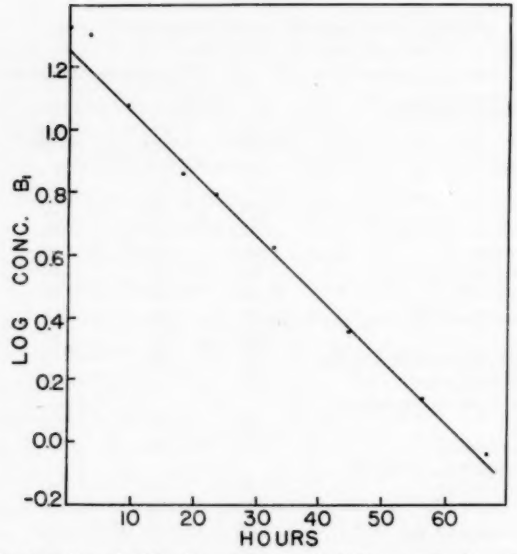

Fig. 1 Destruction of thiamine at 37°C. (initial concentration, 20 mg/kg of purified diet).

TABLE 2

Stability of thiamine in a purified ration under normal feeding conditions

STORAGE CONDITIONS	PER CENT THIAMINE [1] REMAINING		
	Diet		
	1	2	3
2 days in refrig.	100	98	105
6 days in refrig. 1 day in food cup	52	49	62
8 days in refrig. 1 day in food cup	42	47	48
22 days in refrig. 1 day in food cup	33	38	29
23 days in refrig.	42	50	48

[1] Initial concentration of added thiamine: diet 1, 1.5 mg/kg; diet 2, 1.0 mg/kg; diet 3, 0.5 mg/kg.

reaction when the temperature was controlled. Storage of the diet at 37°C. resulted in a very rapid rate of destruction, 68% in one day, and the logarithm of concentration plotted against time yielded the straight line characteristic of a first order reaction. The same percentage of thiamine disappeared from the diet whether the initial concentration of thiamine in the diet was 2 or 20 μg/kg (table 1, diets 1 and 2).

Under normal feeding conditions, when rations were stored in the refrigerator except during the feeding period (about one-half hour per day), the rate at which thiamine disappeared was also quite rapid (table 2). Samples of diet were taken from the refrigerator for analysis and at the same time clean, dry food cups were filled and placed in the animal cages, and the part not eaten after 24 hours was analyzed for thiamine. After 6 to 8 days in the refrigerator and one day in the food cup, about 50% of the thiamine had disappeared.

Effect of fat

Fat-soluble constituents of the diet were studied because of the known sensitivity of certain of the other vitamins to rancidity. Thiamine proved to be somewhat more stable in a fat-free diet than in the basal diet which contained 5% of corn oil (table 1, diets 5 and 7; table 3, diets 1 and 6) and the difference was particularly evident after prolonged storage at low temperatures. But at room temperature the losses of thiamine were still very great in the fat-free diet. Thiamine stability varied somewhat with the nature of the dietary fat, being greater when the fat was an hydrogenated vegetable oil [2] (table 1, diet 9), than when corn oil was used (diet 5), while linseed oil appeared to hasten thiamine destruction (table 1, diets 13 and 15). However, a three-fold increase in the level of α-tocopherol did not improve the stability of thiamine (table 1, diets 5 and 11), while exposure of the diet to light failed to hasten thiamine destruction

[2] Crisco.

(table 1, diets 26 and 27). It would therefore appear that rancidity was not the primary cause of the instability of thiamine in these diets.

Effect of salts

The omission of the salt mixture from the diet decreased the rate of thiamine destruction (table 1, diets 13 and 18, 20 and 21), the effect being particularly evident during the early stages of the storage period. Even in the absence of salts, however, destruction of thiamine was appreciable. The omission of both fat and salts from the diet resulted in somewhat greater stability (table 1, diet 3), although stability in this diet was not absolute. Incidentally, thiamine proved to be completely stable when mixed with sucrose or with sucrose and the other vitamins. When the diet contained salts that had been oxidized with hydrogen peroxide, thiamine destruction was hastened (table 1, diets 13 and 17).

Effect of ascorbic acid and certain other reducing agents

Certain water-soluble anti-oxidants proved to be very effective in preserving thiamine in laboratory diets. Ascorbic acid was one of the best of these. At a level of 5 gm of ascorbic acid per kilogram of diet, thiamine was completely stable for three weeks at room temperature (table 1, diet 4), while at lower concentrations the degree of protection was proportional to the concentration of ascorbic acid (table 3). In many experiments (table 1, diets 6, 8, 10, 12, 14, 16, 19), 500 mg of ascorbic acid per kilogram greatly increased the stability of thiamine, while a concentration of 50 mg of ascorbic acid per kilogram preserved thiamine completely for 44 days in the refrigerator (table 3, diet 2). The proportion of ascorbic acid remaining in the ration was roughly similar to the amount of thiamine remaining (table 3). The protective effect of ascorbic acid was also noted in diets that contained no salts (table 1, diets 18 and 19) or no fat (table

1, diets 7 and 8; table 3, diets 6 and 7). Hydroquinone at 500 mg/kg definitely retarded the disappearance of thiamine (table 3, diets 8 and 9); cysteine at 1 gm/kg was somewhat less active (table 3, diet 10).

TABLE 3

Effect of certain water-soluble anti-oxidants on the rate of thiamine destruction

NO.	CHANGES IN BASAL RATION [1]	PER CENT THIAMINE [2] REMAINING						PER CENT OF ASCORBIC ACID REMAINING AFTER 40 DAYS	
		Refrig.			Room temp.				
		1–2 days	44 days	52 days	7–10 days	17 days	44 days	Refrig.	Room temp.
1	None	102		33	26	7			
2	50 mg ascorbic acid/kg	102		98	64	20			
3	100 mg ascorbic acid/kg	102	93		93	31	10		39
4	500 mg ascorbic acid/kg	98	91		98	68	50	97	49
5	1.5% sulfasuxidine	98	91		74	45	17		
6	No fat	90		82	61	45			
7	No fat + 500 mg ascorbic acid/kg	94	74		98	54	39	64	34
8	None	90	30 [3]		4				
9	500 mg hydroquinone/kg	88	70 [3]		38				
10	1 gm cysteine/kg	92	40 [3]		11				

[1] Diets directly comparable are 1–7 and 8–10.

[2] Initial thiamine concentration in all diets was 2 mg/kg.

[3] Assay values for diets 8–10 were obtained on the 28th day.

Effect of certain physical factors

Storage of the diets under nitrogen retarded the rate at which thiamine disappeared (table 1, diets 28 and 30), although it did not protect thiamine completely. When the ration was desiccated over CaO *in vacuo,* the thiamine was completely stable for two weeks at room temperature (table 1, diet 25). Desiccation over CaO in the presence of air also markedly retarded the rate of thiamine destruction (table 1, diets 28 and 29), and under these conditions thiamine was actually somewhat more stable than when the ration was stored under nitrogen (table 1, diet 30). Thus it appeared

that the destruction of thiamine might be taking place in solution in films of water on the ration particles. Pure, dry thiamine is known to be stable for long periods of time in ordinary reagent bottles.

Glycerol was incorporated into the diet as a material that might form a non-aqueous film over the ration particles, and this material proved to be a very effective preserving agent (table 1, diets 28, 33, and 34).

Effect of sulfa drugs and antibiotics

In agreement with the work of Lyman and Elvehjem ('51), sulfasuxidine retarded the destruction of thiamine (table 3, diet 5), although it was less effective than ascorbic acid on a weight basis. Penicillin and aureomycin were without any effect (table 1, diets 22 and 23) on the stability of thiamine.

Stability of bound thiamine

Dried brewers' yeast or dried pork kidney was incorporated into the ration at a level such that the thiamine content of the rations was approximately 2 mg/kg, and the thiamine content of these diets was determined by the same procedure used for other diets except that the enzymatic digestion step was included. Under these conditions, thiamine in dried yeast and dried kidney was completely stable for two weeks at room temperature (table 1, diets 31 and 32).

Effect of thiamine instability on the growth of rats

As a measure of the effect of thiamine losses on growth, 45 weanling rats divided into groups of 5 were fed rations containing limiting levels of thiamine. For some groups, the thiamine was stabilized by the addition of 500 mg of ascorbic acid per kilogram or 1% of glycerol. The weights of the rats after 4 weeks (table 4) showed that growth was approximately proportional to the amounts of thiamine in

the rations; both glycerol and ascorbic acid improved the growth of rats receiving limited amounts of thiamine in a purified ration.

TABLE 4

Growth of rats fed thiamine in the presence or absence of stabilizers

LEVEL OF THIAMINE	AVE. WT. AT 4 WEEKS		
	Unstabilized basal [1]	Basal plus 500 mg ascorbic acid/kg	Basal plus 1% glycerol
μg/gm	*gm*	*gm*	*gm*
0.5	56	67	74
1.0	96	110	160
1.5	138	170	178

[1] Stability data in table 2, fresh ration mixed at 23 days.

DISCUSSION

Since the thiamine requirement of most animals is so critical, it follows that the instability of the vitamin can seriously complicate any investigation in which the biological end-point is the rate of growth. The problem is particularly serious in the bioassay of natural materials in which the bound thiamine of the material being assayed is more stable than the free thiamine used in the control diet; unless precautions are taken, the growth of rats fed a stable unknown will be compared to the growth of others fed a disappearing standard! The marked stabilizing effect of ascorbic acid upon thiamine and the resulting increased rate of growth raise the question of whether past examples of the growth-promoting effects of ascorbic acid in rats or chicks may not have been due to thiamine rather than to ascorbic acid per se or to alleged effects upon folic acid or vitamin B_{12}.

Several of the present results suggest that an oxidation is involved in the conversion of thiamine into a biologically inactive compound not measured by the thiochrome method. The protective effect of water-soluble anti-oxidants is presumably an expression of their activities as reducing agents. Destruction of thiamine was reduced somewhat in nitrogen, but desiccation was even more effective. The results sug-

gest that thiamine destruction takes place in an aqueous medium in a film around the ration particles, and that atmospheric oxygen is the destructive agent. The protective effect of glycerol is thought to depend on a change in the composition of this film. The increased rate of thiamine destruction when salts were included in the ration appears to be catalytic, since it is unlikely that the reaction would be a first order one if any component of the salt mixture reacted with thiamine in any other way.

The effects of various fats on the rate of thiamine loss are also in harmony with the suggestion of an oxidation reaction, since the relatively saturated fat employed [3] showed a preservative effect and the unsaturated oil, linseed oil, hastened destruction. However, fat is probably not the primary cause of thiamine loss, since α-tocopherol and exposure to light did not influence the rate of the reaction, although they are known to alter the rate of fat oxidation.

There do not appear to be any reports in the literature of a protective effect of ascorbic acid or hydroquinone on thiamine; Zima et al. ('41) have reported that the disulfide compound formed from thiamine by oxidation with dilute hydrogen peroxide at pH 7.5 was reduced back to thiamine by cysteine or glutathione but not by ascorbic acid.

SUMMARY

1. Thiamine was unstable in a typical laboratory ration and it disappeared logarithmically with time. The rate of disappearance was greatly influenced by temperature. Considerable destruction occurred under normal feeding conditions, and rats fed diets limiting in thiamine grew better when stabilizing agents were included in the ration.

2. Thiamine was less stable in the presence of fat than in its absence. Linseed oil was more deleterious than corn oil or hydrogenated vegetable oil; α-tocopherol did not affect the stability of thiamine.

[3] See footnote 2, page 213.

3. The stability of thiamine was increased by the addition of ascorbic acid, hydroquinone, cysteine or sulfasuxidine to the ration, or by desiccation or storage under nitrogen. The best stabilizing agent encountered was 1% of glycerol.

4. The addition of penicillin or aureomycin to the ration or exposure of the ration to light did not affect thiamine stability. Stability was decreased somewhat by an ordinary salt mixture, and even more by oxidized salts.

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ENZYMATIC LIBERATION OF AMINO ACIDS FROM DIFFERENT PROTEINS[1]

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The importance of the availability of amino acids has been demonstrated by several workers. Berg and Rose ('29) observed that the frequency of administration of tryptophan to rats fed a tryptophan-deficient ration had a marked influence on their rate of growth. Elman ('39) found that tryptophan and methionine had to be injected at the same time as the other amino acids in order to produce a positive nitrogen balance in dogs. Henry and Kon ('46) and Geiger ('48) concluded from their work that in order for two proteins to supplement each other they had to be fed simultaneously. Melnick et al. ('46) listed the availability of the essential amino acids for tissue anabolism as one of the principal factors which determine the value of food as a source of protein. Geiger ('47) and Geiger and Hagerty ('50) showed that the delayed supplementation of rats with either tryptophan, lysine or methionine resulted in poor growth. Cannon et al. ('47) observed that rats failed to grow when fed 5 of the essential amino acids one hour and the other 5 the next hour. Henderson and Harris ('49) found that a delay of three hours or longer in the feeding of lysine as a supplement to a lysine-low diet interfered with metabolism.

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Both *in vivo* and *in vitro* methods have been employed to determine the availability of amino acids. Each of these methods, although apparently giving somewhat different results, has certain advantages and disadvantages.

In order to study this problem, it was decided first to determine the rate of liberation of amino acids from different proteins employing *in vitro* methods. Although this method may not give a true indication of the *in vivo* availability, it may be useful in detecting any large differences which occur in the liberation of amino acids. Further work is now in progress to determine whether or not these results can be correlated with those obtained by *in vivo* studies.

EXPERIMENTAL

The enzymatic digestion was carried out in a three-necked distillation flask equipped with a stirrer and glass electrode. Constant stirring was employed and a pH reading of within 0.2 pH unit was maintained throughout the digestion period. The pH was adjusted by the addition of acid or base rather than by the use of buffers because of the possible interfering effects of a high salt concentration on the microbiological assays.

Each protein studied was subjected to hydrolysis by crystalline pepsin [2] at pH 2.0, followed by hydrolysis by preparations of defatted and desiccated whole pancreas and duodenal powder [3] at pH 8.0. The first stage of digestion was carried out using a 2.5% crude protein solution with a final concentration of pepsin of 0.025%. The solution was made by placing the protein and pepsin in a volumetric flask and adding HCl (pH 2.0) which had been heated to 37°C. A sample was immediately withdrawn and inactivated and the remainder of the solution placed in the digestion flask.

After three hours of pepsin digestion, the pH was adjusted to 8.0 and the defatted and desiccated whole pancreas and duodenal powder were added. Since in previous stud-

[2] Armour and Company, Chicago, Illinois.
[3] VioBin Corporation, Monticello, Illinois.

ies we had encountered difficulty in getting the enzyme preparations wetted or into fine suspension after adding them as the dried powder, the following procedure was carried out: the dried preparations were homogenized in a Potter-Elvehjem homogenizer with pH 8.0 KOH solution and filtered through a double layer of cheesecloth. Enough of this solution was then added to the digestion flask to bring the concentration of crude protein to 2.0%. The final concentration of the whole pancreas and duodenal powder was 0.20% and 0.10%, respectively. One milliliter of toluene and a few drops of chloroform were added to the digestion flask at the beginning of the experiment.

At the end of the digestion periods the samples were adjusted to pH 10.0 for inactivation of pepsin and pH 2.0 for inactivation of the pancreatic and duodenal enzymes, and then heated for 20 minutes in a boiling water bath. The samples were then filtered through filter paper, adjusted to pH 2.0, made to a definite volume and stored in a frozen state until analyzed.

The proteins used were commercial casein [4] and zein [5] and beef round that had been lyophylized and ether-extracted as described by Sirny et al. ('50).

The extent of liberation of amino acids from an enzyme blank was determined without substrate, using the procedures previously described.

Liberation of the amino acids was followed by the nitrous acid micro-method of Van Slyke ('15), using a 4-minute reaction time, by the manometric micro-ninhydrin procedure (2 ml of carbon dioxide gas) of Van Slyke et al. ('41), and by microbiological assays. The medium of Henderson and Snell ('48), with the modifications described by Violante et al. ('52), was used for all microbiological determinations. The following microorganisms were used for determining the amino acid composition of the enzymatic digest: *Lactobacil-*

[4] Labco washed casein (Borden).

[5] Nutritional Biochemical Corporation, Cleveland, Ohio.

lus delbrucckii 3 was used for arginine, *Leuconostoc mesenteroides* P-60 for histidine, isoleucine, lysine and methionine, *Lactobacillus arabinosus* 17-5 for leucine, phenylalanine, tryptophan and valine, and *Streptococcus faecalis* for threonine.

RESULTS AND DISCUSSION

The results of the liberation of amino acids as measured by nitrous acid and ninhydrin procedures are presented in tables 1 and 2. It appears from these results that there is

TABLE 1

Per cent[1] *liberation of amino acids as measured by the nitrous acid micro-method of Van Slyke*[2]

TIME	BEEF	CASEIN	ZEIN
0 Hr. I[3]	10.1	2.87	0.32
0 Hr. II[4]	24.9	16.0	6.35
1 Hr. II	34.4	26.0	14.7
3 Hr. II	39.5	30.8	28.6
5 Hr. II	40.7	32.0	31.7
8 Hr. II	41.9	33.2	32.9
12 Hr. II	43.5	39.1	39.7
24 Hr. II	48.6	42.1	41.6
48 Hr. II	50.2	45.6	45.2
72 Hr. II	55.7	47.2	48.8

[1] One hundred per cent liberation of the amino acids from the proteins was based on a 15-hour hydrolysis with 3N HCl at 121°C.

[2] All values are corrected for an enzyme blank.

[3] Pepsin stage of digestion; values obtained prior to any enzymatic action.

[4] After three hours of pepsin digestion and at the beginning of digestion with whole pancreas and duodenal powder.

little difference in the rate of liberation of amino acids from these three proteins, with a few possible exceptions. For example, the amount of amino acids liberated from zein during the pepsin stage of digestion, when measured by the nitrous acid method (table 1), appears to be less than the amount liberated from the other two proteins. On the other hand, the liberation of amino acids from zein and casein when measured by the ninhydrin procedure appears to be less than that from beef during the pepsin stage of digestion.

These differences may be due to the differences in the preparation of the proteins. This fact was emphasized by Albanese et al. ('49) when they observed that the liberation of amino acids from zein was less than that from casein.

Differences in the rates of hydrolysis of different proteins have been observed previously in this laboratory when constant stirring was not employed. Apparently the stirring increases the rate of hydrolysis of insoluble proteins more than it does the rate of those which are readily dissolved or easily suspended in solution.

TABLE 2

Per cent liberation of amino acid as measured by the manometric micro-ninhydrin procedure of Van Slyke et al.[1]

TIME	BEEF	CASEIN	ZEIN
0 Hr. I	1.19	0	0
0 Hr. II	6.94	1.54	1.95
1 Hr. II	14.6	13.5	12.2
3 Hr. II	21.0	17.3	20.6
5 Hr. II	28.9	18.9	23.3
8 Hr. II	29.8	19.7	27.2
12 Hr. II	31.5	24.6	29.7
24 Hr. II	37.1	27.8	40.6
48 Hr. II	37.6	31.8	44.2
72 Hr. II	49.4	41.0	47.0

[1] See footnotes for table 1.

The data for the microbiological assays are presented in table 3. Assays were run on samples collected at 10 intervals of time, but for simplicity only 5 are presented. These values are indicative of the rates of hydrolysis and also the extent, except for a few amino acids which did become more microbiologically available after 24 hours.

There were some differences in the rate of liberation of the amino acids from the three proteins during the pepsin stage of digestion. The proteins of beef appeared to be broken down into microbiologically available amino acids more rapidly than casein or zein, with arginine, threonine and valine being exceptions. The values in tables 1 and 2 also

TABLE 3

Per cent liberation[1] of microbiologically available amino acids from different proteins[2]

HOURS	0 – I			0 – II[3]			1 – II			5 – II			24 – II		
Protein[4]	B	C	Z	B	C	Z	B	C	Z	B	C	Z	B	C	Z
Arginine	10.3	20.9	8.89	76.6	71.5	83.3	84.7	70.2	86.2	89.5	76.2	88.9	84.7	78.6	80.6
Histidine	1.15	0	0	7.14	1.56	0	25.7	17.2	29.4	37.1	31.3	41.2	48.6	37.5	55.9
Isoleucine	0.91	0	0	15.9	2.00	0	42.1	14.0	10.3	56.8	20.0	19.9	73.9	26.7	28.8
Leucine	1.61	2.80	1.35	42.6	25.0	32.5	72.0	66.0	58.8	78.0	70.5	63.7	80.0	70.5	83.5
Lysine	1.37	0	...	16.2	9.42	...	64.1	36.5	...	78.6	45.3	...	92.3	54.1	...
Methionine	1.67	0	0	22.9	2.86	0	52.2	21.4	19.6	70.9	30.0	41.3	85.4	42.8	52.2
Phenylalanine	1.11	1.91	1.25	50.0	25.4	7.81	75.0	49.2	82.0	86.1	56.3	87.4	84.8	62.6	90.6
Threonine	1.78	0.89	4.00	53.3	31.1	60.3	80.0	60.0	76.9	83.3	64.4	78.4	82.3	64.3	81.8
Tryptophan	0	3.13	0	38.5	38.5	0	61.6	65.4	0	73.2	73.1	50.0	73.2	77.0	100.0
Valine	3.64	0.52	2.67	40.9	17.5	46.7	65.9	39.6	75.0	76.2	41.5	96.7	81.9	50.6	96.7

[1] One hundred per cent liberation of the amino acids from the proteins was determined by hydrolysis with 3N HCl for 15 hr. at 121°C. in all cases except tryptophan, where 4N KOH for 15 hr. at 121°C. was used.

[2] All values are corrected for an enzyme blank.

[3] After three hours of pepsin hydrolysis and at the beginning of the second stage of hydrolysis.

[4] B = beef; C = casein; Z = zein.

indicate that the proteins of beef were hydrolyzed more rapidly than the other two proteins. The differences in the rates of liberation of amino acids from the proteins of beef and the other two proteins may be due to the fact that the beef was prepared in a manner which would tend to preserve the intracellular proteolytic enzymes.

Arginine was liberated very rapidly into a microbiologically available form from the three proteins during the pepsin stage of digestion. This phenomenon was also observed by Hankes et al. ('48) and is being studied further at the present time.

During the second stage of digestion several differences in the rates of liberation of amino acids from the three proteins were evident. Again, as in the pepsin phase of digestion, the amino acids appeared to be liberated in a microbiolgically available form from the proteins of beef more rapidly than from the other proteins. Isoleucine and methionine were liberated much more slowly from casein and zein than from beef. Williams and Elvehjem ('49), using the activity of liver xanthine oxidase as a criterion for amino acid availability, observed that methionine in dietary casein was not readily available to the rat.

By comparing the liberation of amino acids within each protein it is evident that histidine is liberated more slowly from beef than are the other 9 amino acids. Kuiken and Lyman ('48), using an *in vivo* method, found all of the essential amino acids of roast beef completely available. Histidine, isoleucine and methionine appear to be liberated from casein less readily than the other amino acids. The values for the per cent liberation of amino acids from casein in the present study agree favorably with those of Hankes et al. ('48), with the exception of methionine and valine, in which cases they found a greater percentage liberation. The per cent liberation of amino acids from zein indicates that isoleucine and possibly methionine and histidine are liberated less rapidly than the other amino acids. Since it is not known at what rate each amino acid should be liberated for optimum utiliza-

tion, it is impossible to conclude from these studies which of the proteins, if any, are hydrolyzed at an optimum rate. Silber et al. ('46) and Silber and Porter ('49) found greater utilization of injected amino acid mixtures with a slower rate of infusion; therefore, rapid liberation does not necessarily mean greater utilization.

SUMMARY

1. It was found that the rate and extent of liberation of amino acids from beef, casein and zein, when measured chemically, were approximately the same, with beef being hydrolyzed slightly more rapidly than the other two proteins.

2. Wide variations occurred in the liberation of amino acids from the proteins when measured microbiologically; however, it again appeared that beef was hydrolyzed slightly more rapidly than the other two proteins.

3. Microbiologically available arginine appeared to be liberated from all proteins studied very rapidly during the pepsin stage of digestion.

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NUTRITIONAL VALUE OF PLANT MATERIALS

VIII. LACTATION FAILURE OF RATS FED DIETS CONTAINING PURIFIED SOYBEAN PROTEINS [1, 2]

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ONE FIGURE

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Previous papers from this laboratory (Schultze, '50a, b) recorded observations on the growth and reproductive performance of rats maintained on diets containing organic ingredients of synthetic and plant origin. In some of these diets the only source of amino acids was a purified soybean protein supplemented with DL-methionine. Such a diet (ration S_3) supported satisfactory post-weaning gains; the first litter of rats reared by mothers consuming this diet had a pre-weaning mortality of 18.7%, which rose to 50.4% in the second litter. This paper summarizes further observations made during the past three years on the reproductive performance of succeeding generations of rats maintained on the same type of ration. The evidence presented below indicates that the feeding of certain purified soybean protein preparations leads to a gradual exhaustion of the ability of the maternal organism to support the pre-weaning survival of her young. The nature of this nutritional disturbance is not understood at the present time, but it appears that

[1] Paper 2891, Scientific Journal Series, Minnesota Agricultural Experiment Station.

[2] A preliminary report of these studies was presented at the 18th International Physiological Congress, Copenhagen, August 15, 1950. Abstracts of Communications, 18th International Physiological Congress, page 440.

factors closely associated with dietary proteins are intimately concerned with the prevention, or cure, of this condition.

EXPERIMENTAL

Animals

The animals used in these experiments were the first filial and subsequent generations of rats raised since weaning on ration S_3 containing 24% of purified soybean protein (groups 13 and 14, table 2, Schultze, '50b). From each generation of rats suitable survivors were raised to maturity on the same type of ration and used for observation of their reproductive performance.

Since (a) rats appear to be rather slowly depleted of the factors involved, (b) there seems to be a marked carry-over from the maternal organism to the young, and (c) the rations used may be only partially deficient in the factors concerned, the technique of using successive generations of rats maintained on the same basal ration is probably justified, and necessary. The females were bred when 10 weeks old with males maintained on the same rations. Shortly before parturition and during lactation the females were housed individually in cages containing clean wood shavings. The young were examined for abnormalities and weighed daily until they were weaned at 21 days of age. As in our previous studies, the size of the litter was never reduced and the females were returned to the breeding cages immediately after weaning or after the loss of a litter. By the technique described, rats have been maintained for 10 successive filial generations on rations composed essentially of ingredients of synthetic and plant origin and salts.

Rations

The ingredients and composition of the rations were essentially the same as previously described (Schultze, '50a). The only differences between rations S_3, S_{13}, and S_{20}, as shown in table 1, were in the origin of the purified soybean

protein and in the vitamin B_{12} content. The soybean proteins were purified as previously described (Schultze, '50a) from soybean sodium proteinate [3] or from soybean "alpha protein." [4] This purification involved two precipitations from an aqueous medium, followed by 72 hours' extraction with three changes of boiling ethanol. In the case of the "alpha protein" this treatment removed most of the sulphite which has been a complicating factor in some nutrition experiments in which the crude product has been used.

TABLE 1

Composition of rations

RATIONS	S_3	S_{13}	S_{20}
	gm	*gm*	*gm*
Purified soybean protein	234.4 [1]	234.4 [1]	234.4 [2]
DL-Methionine	5.6	5.6	5.6
Salts [3]	40.0	40.0	40.0
Vegetable fat (Crisco)	70.0	70.0	70.0
Corn oil + vitamin mixture [4]	10.0	10.0	10.0
Sucrose + vitamin mixture [5]	20.0	20.0	20.0
Sucrose	620.0	620.0	620.0
		μg	*μg*
Vitamin B_{12} [6]	none	40	40

[1] Prepared from soybean sodium proteinate, Archer Daniels Midland, Minneapolis.
[2] Prepared from soybean "alpha protein," The Glidden Co., Chicago, Illinois.
[3] Schultze ('50a).
[4] Schultze ('50a). Since May, 1950, synthetic vitamin A acetate was substituted in equivalent units for the fish liver oil concentrate previously used.
[5] Schultze ('50a), including biotin.
[6] Merck and Co., "Cobione."

Unfortunately, it was not possible to use the same batch of soybean sodium proteinate throughout the three years of this study. Different batches of this starting material, identified in this paper by Roman numerals, exhibited some differences in physical and chemical properties and, what is more important, in some cases pronounced differences in nutritional qualities. It has not been possible to ascertain

[3] Archer Daniels Midland Co., Minneapolis, Minnesota.
[4] The Glidden Company, Chicago, Illinois.

whether such differences in nutritional qualities reflect variations in the chemical composition of the soybeans or whether variations in the manufacturing procedure are solely responsible. Evidence that the latter are partly involved will be indicated below. The concentration of the essential amino acids in the purified protein which was prepared from sodium proteinate I has been previously reported (Schultze, '50a). The apparent digestibility of the dietary nitrogen containing this purified protein was determined with 6 mature female rats whose weights were maintained constant by feeding the appropriate amount of ration S_{13} by means of a stomach tube. After a preliminary two-day period, the fecal nitrogen excretion, collected over a period of 4 days, indicated a mean apparent digestibilty of the dietary nitrogen of 92.5%.

RESULTS AND DISCUSSION

The data summarized in table 2 present evidence concerning three important features of the deficiency syndrome described in this paper.

1. The pre-natal and pre-weaning nutritional history of the mothers had a pronounced effect on the mortality of the young subsequently born. The mothers in groups 1 and 2 consumed ration S_3 from the time they were weaned at 21 days of age and throughout their subsequent period of growth and reproduction. Their mothers, however, had been maintained throughout life on ration S_2 (Schultze, '50a) which differed from ration S_3 only by containing the unpurified soybean sodium proteinate. The mortality of the young born in the first litter (group 1) was relatively low, probably as a result of the carry-over of some dietary factor accumulated by the mothers during their life *in utero* and before weaning. In contrast to this, when mothers were raised from parents maintained on ration S_3, the pre-weaning mortality of the young was extremely high (group 3).

2. When suitable preparations of a purified soybean protein were used in the maternal diet, a progressive decrease in the survival of the young of successive litters was ob-

served. The data pertaining to groups 1 and 2 of table 2, and the data of table 3 present evidence for a progressive depletion from the maternal organism of some dietary factors required for the survival of the young. It is recognized that the decreased survival of the young might also reflect an increased maternal requirement for these factors with advancing age and with the production of successive litters of young. Only in a few instances has there been a decreased

TABLE 2

Effect of previous maternal dietary history and of vitamin B_{12} on mortality of young rats

(Ration S_2. Purified soybean protein I)

GROUP NO.	LITTER SEQUENCE	HISTORY OF MOTHERS	RATION OF		VIT. B_{12} PER KG OF MOTHER'S RATION	NO. OF LITTERS	MEAN NO. OF YOUNG PER LITTER	PER CENT MORTALITY TO 21ST DAY
			Grandmothers	Mothers				
					μg			
1	1st	Same mothers [1]	S_2 [4]	S_2	None	30	7.9	37.0
2	2nd		S_2 [4]	S_2	None	30	7.7	66.4
3	1st	[2]	S_2	S_2	None	52	6.8	93.5
4	2nd	[2,3]	S_2	S_2	100	13	7.6	92.4
5	3rd	[2,3]	S_2	S_2	100 2–9 days pre-partum 100 31–59 days pre-partum	13	7.1	86.9

[1] See Schultze ('50b), table 2, groups 13 and 14.
[2] Mothers were F_1 generation of group 1.
[3] Mothers originally in group 3.
[4] Protein of ration S_2 was unpurified soybean sodium proteinate.

mortality of the young of successive litters borne by the same mothers when no changes were made in the diet.

3. The feeding of high levels of vitamin B_{12} for as many as 59 days prepartum had no curative effect on this deficiency syndrome after it was established in an animal (groups 4 and 5, table 2).

In view of these observations made during the early phases of this study, the basal ration used for subsequent experiments (ration S_{13}) was fortified with 40 μg of vitamin B_{12}

per kilogram of ration. The details of the reproductive performance of the rats maintained on ration S_{13} since birth are illustrated in table 3. Again there was a high pre-weaning mortality which increased with the second litters born. While the prophylactic feeding of vitamin B_{12} obviously did not prevent a high mortality of young, the addition of this compound to the ration may have exerted some beneficial effect. This is indicated by the fact that with ration S_{13} the mortality of the young during their first 4 days of life was usually less than 15%, whereas with many vitamin B_{12}-deficient rations it ranged from 40 to 60% (Schultze et al., '52). In

TABLE 3

Mortality of young

(Ration S_{13}, purified soybean protein preparation Ia)

	FIRST LITTER	SECOND LITTER
No. of young born	285 (38)[1]	340 (37)[1]
Mortality 0–21st day, %	68.2	80.7
Mean 21-day weight per survivor, gm	38.6	33.6
Mean weight change of mothers during lactation, gm	+ 9.1	— 2.4

[1] Number of litters in parentheses.

addition, the pre-weaning mortality in the first litters born never approached the extremely high values of over 90% observed in the absence of vitamin B_{12} (table 2, group 3).

The deficiency of rats whose litters showed close to 100% mortality can be alleviated by dietary means. As shown in table 4, the substitution, at parturition, of soybean oil meal (expeller type) for the purified soybean protein in the ration of mothers whose previous litters had suffered almost 100% mortality resulted in a pronounced curative effect even during this short interval. It is evident, therefore, that the soybean contains one or more nutrients which by suitable purification can be at least partly removed from the protein. In the absence of these nutrients rats are unable to rear their young.

This study indicates that the nutritional factors involved are closely associated with dietary protein. In spite of the fact that the same method was employed for the purification of different commercial preparations of soybean protein available, marked differences in the biological properties of the

TABLE 4

Effect of soybean oil meal on mortality of successive litters (Change of ration at birth of 5th litter)

	RATION S_{13}	RATION S_7 [1]
Source of protein	Purified soy prep. Ia	Soybean oil meal
No. of mothers	10 (4th litters)	10 (5th litters)
No. of young born	92	79
Mortality 0–21 days, %	97.8	51.9

[1] Schultze et al. ('52). This ration contained 485 gm of expeller type soybean oil meal and 369.4 gm of sucrose in lieu of 234.4 gm of purified soybean protein and 620 gm of sucrose.

TABLE 5

Mortality of young

(Ration S_{20} containing purified "alpha protein"; consecutive litters from same rats)

	1ST LITTER	2ND LITTER	3RD LITTER
No. of young born	223 (33)[1]	267 (33)[1]	278 (32)[1]
Mortality 0–21st day, %	42.3	37.1	38.9
Mean 21-day weight per survivor, gm	34.5	34.0	34.0
Mean weight change of mother during lactation, gm	+ 10.7	0.0	— 6.2

[1] Number of litters in parentheses.

purified products were observed. As shown in table 5, the purified protein prepared from "alpha protein," [5] when used as a component of ration S_{20}, supported for three consecutive litters the survival of about 60% of the young. The mortality of the young during the first 4 days of life was less than 5% in this experiment.

[5] See footnote 4, page 233.

Purified preparations made from various batches of commercial soybean sodium proteinate supplied by the same manufacturer [6] gave variable results. According to the data summarized in table 6, the purified product made from preparation VII gave essentially the same response as that obtained with preparations I and Ia, respectively (tables 2 and 3). In contrast, the purified product prepared from preparation VIIIa had a pronounced curative and prophylactic effect and supported satisfactory survival of the young. Information supplied by the manufacturer failed to disclose

TABLE 6

Mortality of young rats

(Ration S_{22}; effect of different soybean protein preparations)

LITTER SEQUENCE	PROTEIN PREPARATION				
	VII	VII	VIIIa	VIIIa	IVa
	Same mothers 1st	Same mothers 2nd	2nd [1]	1st and 2nd	1st and 2nd
No. of young born	149 (23)[2]	61 (8)	170 (21)	319 (45)	170 (23)
Per cent mortality to 21 days	57.7	77.1	20.0	14.0	31.6
Mean 21-day weight of young, gm	33.8	33.5	33.7	35.9	16.7
Mean weight change of mother during lactation, gm	+ 8.7	+ 6.2	+ 3.0	+ 0.5	— 39.2

[1] Including 15 litters from mothers who had their first litters while consuming protein preparation VII.

[2] Number of litters in parentheses.

differences in the processing procedure which could account for the different biological effect. High mortalities of the young have also been observed with rations containing purified soybean protein prepared from 4 other soybean sodium proteinate preparations. These data, covering over 200 litters, are not presented here.

Of special interest are the results obtained with the purified product made from soybean sodium proteinate preparation IVa. According to the manufacturer, in the processing of this starting material, the dissolved soybean proteins were heated at 138°F. for two hours in a solution

[6] See footnote 3, page 233.

made alkaline to pH 11.0 with lime. This solution was subsequently neutralized to pH 7 and spray-dried. The purified protein prepared from this product was fed as a component of ration S_{13} to weanling rats and to mature rats previously raised on ration S_{13} containing the protein derived from preparation VIIIa. As shown in table 6, the pre-weaning mortality of the young born to these rats was not excessively

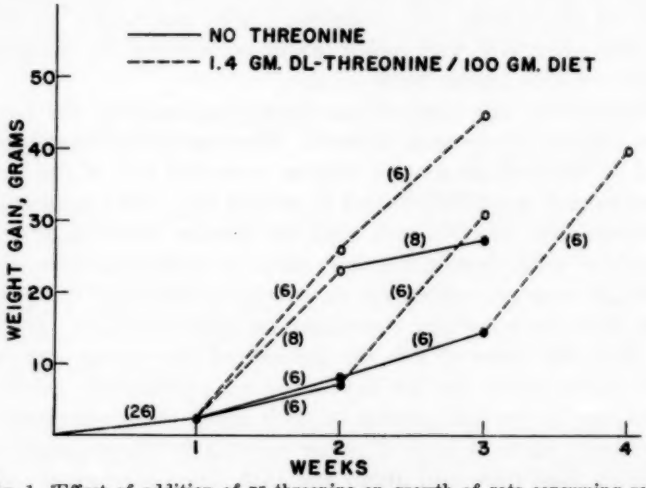

Fig. 1 Effect of addition of DL-threonine on growth of rats consuming ration S_{13}. The purified soybean protein was made from preparation IVa. Number of rats in parentheses.

high, but the mothers suffered a severe loss of weight, particularly during the early stages of lactation. Although apparently normal at birth, the young born to these mothers grew very slowly and weighed in some cases as little as 10 gm at 21 days of age. When the same ration was fed to weanling rats, their growth was very slow unless the diet was supplemented with 1.4% of DL-threonine, as shown in figure 1. The proteins derived from preparation IVa were evidently deficient in L-threonine, perhaps through racemization incurred during the alkaline treatment used in the proc-

essing. In spite of this partial amino acid deficiency, mature animals made normal weight gains during pregnancy, and did not lose weight unless subjected to the additional stress of lactation. Whether the slow pre-weaning growth of the young reflects a deficiency of the amount of milk secreted or of the threonine content of the milk cannot be decided on the basis of the available evidence. It is of interest to note, however, that the mortality of the young, in spite of this amino acid deficiency, was not nearly as high as that observed with other diets containing an adequate supply of this amino acid.

Throughout this study it has been observed that the young were apparently normal at birth. The number of young born dead in the various groups seldom exceeded 1% of the total. Close visual inspection failed to reveal any gross anatomical abnormalities and showed that the young received a large supply of milk during the first three to 4 days of life. Cannibalism was an extremely rare occurrence, and the young that died were seldom consumed by their mothers. During the first few days of life the growth of the young was normal. After about the 5th day following parturition, however, from one to several young in each litter were observed on successive days to have little or no milk in the stomach and between that time and about the 14th day post-partum most of the young died. However, in most cases, the young that survived developed normally as shown by their weight at 21 days of age (tables 3, 5, and 6).

The post-weaning weight gains, the appearance, and the subsequent breeding performance of the surviving rats were normal in every respect for this strain of rats. Thus, the mean weight gains during the 6 weeks' post-weaning period of 34 female rats of the F_{10} generation were 112.6 gm $\pm$ 1.7 [7] and those of 10 males were 173.8 gm $\pm$ 3.0.[8] These figures are almost identical with those reported previously (Schultze, '50a) for rats of the F_2 and F_3 generations, which

[7] Standard error of the mean.

[8] See footnote 7.

were the first animals maintained on rations containing the purified soybean protein. It appears, therefore, that these purified rations, composed of ingredients of synthetic and plant origin and of salts, are adequate to meet the nutritional requirements of the rat except during the stress imposed by lactation. In this connection, it is interesting to note that except in the instance cited in table 6 the mothers usually gained weight during the period of lactation in spite of the fact that they were unable to raise their young as a result of dietary inadequacy. The diets used were adequate to meet

TABLE 7

Mortality of young reared by foster mothers

	RATION OF MOTHERS	
	S_3, Purified	S_7, Soybean oil meal [1]
No. of mothers	6	6
No. of own young nursed	19	25
No. of foster young nursed	21	21
Pre-weaning mortality of own young, %	100	12.0
Pre-weaning mortality of foster young, %	100	9.5

[1] See footnote, table 4.

the stress of pregnancy because the mothers usually weighed from 20 to 30 gm more after parturition than at the time of conception.

Evidence that the nutritional disturbance recorded in this paper manifests itself through inadequate quantity or quality of the milk secreted or through a combination of both of these factors is shown in table 7. For this experiment, about half of the young from mothers maintained on ration S_3 were transferred shortly after birth to mothers maintained on ration S_7 (Schultze et al., '52) and vice versa. Ration S_7 contained an equal amount of total nitrogen in the form of an expeller type of soybean meal in lieu of the purified soybean protein present in ration S_3. The mothers fed ration S_3 were unable to rear their own or their foster young, while those fed S_7 successfully weaned about 90% of their own

and 90% of the foster young. These data indicate, therefore, that the mothers fed the deficient ration S_3 are unable to produce milk of adequate quantity or quality, or both, to permit the survival of the young. The same applies to ration S_{13}, which contains added vitamin B_{12}. The latter is ineffective in preventing the lactation failure. If the young were born with functional impairment established during intrauterine development, such impairment could evidently be overcome by an adequate milk supply, as was the case with the milk produced by mothers maintained on ration S_7.

No conclusions can be drawn from the evidence presented here concerning the nature of the dietary deficiency described. Its relation to other dietary factors concerned with the reproduction of rats will be discussed in the following paper in which the effects of various supplementary procedures will be documented.

SUMMARY

1. The feeding to rats of rations containing salts and components of synthetic and plant origin, including certain preparations of purified soybean protein, leads to a high pre-weaning mortality of the young.

2. The cause of this pre-weaning mortality of the young appears to be lactation failure.

3. The nutritional deficiency cannot be corrected by curative or prophylactic feeding of vitamin B_{12}. Crude soybean oil meal added to the maternal diet at parturition has a curative effect.

4. The nature of the factor or factors responsible for this deficiency is unknown. Great variations have been encountered in the extent to which commercial preparations of soybean protein can be purified so as to establish this dietary deficiency.

5. Rats have been maintained on purified rations containing salts and components of synthetic and plant origin for 10 filial generations without evidence of functional impairment except for a high incidence of lactation failure.

6. A partial threonine deficiency of the maternal diet was responsible for subnormal weaning weights of the young and for a marked loss of weight of mothers during lactation.

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NUTRITIONAL VALUE OF PLANT MATERIALS

IX. THE EFFECT OF VARIOUS SUPPLEMENTS ON LACTATION FAILURE OF RATS FED DIETS CONTAINING PURIFIED SOYBEAN PROTEINS [1]

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According to the evidence summarized in the preceding paper (Schultze, '53), a high incidence of lactation failure can be induced in rats maintained for more than one generation on diets composed of salts and ingredients of synthetic and plant origin in which highly purified soybean proteins and DL-methionine furnish the only source of amino acids. It was also shown that this dietary deficiency can be cured, at least in part, by the substitution, at parturition, of soybean oil meal for the purified proteins of the diet. Since the nature of the factors involved in the prevention or cure of this deficiency was not recognized, it was of interest to study the effect of various supplements to the diet on this syndrome. The present paper presents further evidence that the lactation failure is the result of a progressive depletion of the maternal organism, that it is not subject to spontaneous remission without change of diet, and that it can be prevented or cured through dietary means.

EXPERIMENTAL

Details concerning the dietary history and the handling of the rats used in these experiments have been described in the preceding paper. The rats used in all of the experi-

[1] Paper 2890, Scientific Journal Series, Minnesota Agricultural Experiment Station.

ments were offspring of parents who had been maintained throughout their lives on ration S_{13}. For curative experiments, only those rats were used whose preceding litters (one or two) had shown a high incidence of pre-weaning mortality. In the prophylactic experiments, however, the dietary supplement was fed to the rats from the time they were weaned.

Throughout this study ration S_{13}, which contains purified soybean proteins and DL-methionine as described in the preceding paper, was used as the control reference diet. Ration S_{14} was prepared by the addition of 100 mg of orotic acid, synthesized essentially according to the method of Nyc and Mitchell ('47), per kilogram of ration S_{13}. Ration C_2 contained per kilogram 240 gm of "vitamin-free" casein [2] in lieu of the purified soybean proteins and the DL-methionine of ration S_{13}. Ration A_1 was made by substituting 257.0 gm of a protein of animal origin [3] plus 2.8 gm of DL-methionine for the 234.4 gm of purified soybean proteins, 5.6 gm of DL-methionine and 19.8 gm of sucrose contained in 1 kg of ration S_{13}. The total nitrogen content and the calculated [4] methionine content of rations S_{13} and A_1 were the same. Since it was impossible to use throughout this study purified soybean proteins derived from the same starting material, the various preparations have been identified in each experiment with Roman numerals in order to permit comparison with the experiments recorded in the preceding paper.

RESULTS

The effect of a grass juice concentrate on lactation failure

For this experiment, two groups of mature females were selected who had been fed ration S_{13} throughout their lives

[2] Labco casein, The Borden Company, New York.

[3] "B Protein," Lot 83992, The Wilson Laboratories, Chicago, who furnished data on the composition of the product. It is a mixture of brain and spinal cord extracted with boiling alcohol and with acetone at room temperature.

[4] See footnote 3.

and whose preceding two litters had incurred a high incidence of pre-weaning mortality. After loss or weaning of the second litter their diet was supplemented with 2% or 10% of a water-soluble concentrate prepared from young cereal grasses. According to the manufacturer [5] of this product it contained 50% of solids, 0.8% of nitrogen, 10% of ash and the following vitamins in terms of micrograms per gram: riboflavin 40, nicotinic acid 90, pantothenic acid (free) 25, folic acid *(S. faecalis)* 20, pyridoxine 10, biotin 0.4. As shown by the data summarized in table 1, the inclusion of as much

TABLE 1

Effect of grass juice concentrate on mortality of successive litters

(Ration S_{13}, purified soybean protein preparation Ia, 15 mothers)

GRASS JUICE CONCENTRATE, %	0 FIRST LITTER	0 SECOND LITTER	10 THIRD LITTER
No. of young born	137	161	142
Mortality in 21 days, %	65.6	100	94.4

as 10% of this grass juice concentrate into ration S_{13} was practically devoid of curative effect on the lactation failure of the rats. The results of a similar experiment in which 2% of the grass juice concentrate was fed to 11 mothers during the third pregnancy and lactation were likewise negative. The data presented in table 1 illustrate two important features concerning the syndrome under study:

1. The maternal organism is progressively depleted of the preventive factors.

2. A spontaneous improvement of lactation performance without a change of diet during the production of successive litters is a rare occurrence.

The effect of folic acid on lactation failure

Other investigators (Sica et al., '48; Nelson and Evans, '48; Williamson, '49) have shown that restriction of the

[5] The Cerophyl Laboratories, Kansas City.

maternal organism in folic acid increases the pre-weaning mortality of young rats, presumably through impairment of lactation. The amount of folic acid (0.20 mg per kilogram) added to ration S_{13} which was used in these and preceding experiments might conceivably have been somewhat low to meet the requirements for adequate lactation. The data summarized in table 2 (group 1), however, show that a 25-fold increase in the amount of folic acid in the diet of mothers

TABLE 2

Curative effect of various supplements to the maternal diet on the mortality of young rats

GROUP	RATION	SOYBEAN PROTEIN PREPARATION	SUPPLEMENT ADDED	LITTER SEQUENCE	NO. OF YOUNG BORN	MORTALITY IN 21 DAYS
						%
1	S_{13}	Ia	None	1st	219 (27)[3]	68.4
	S_{13}	Ia	Folic acid, 4.8 mg per kg[1]	2nd	241 (27)	88.0
2	S_{13}	Ia	None	1st	153 (19)	62.7
	S_{13}	Ia	None	2nd	199 (19)	96.0
	C_2	..	Vitamin-free casein in lieu of soybean protein[1]	3rd	120 (19)[4]	35.0
3	S_{13}	IIa, VI	None	1st	84 (10)	60.7
	S_{13}	VI, V	None	2nd	73 (8)	82.1
	A_1[2]	..	Protein of animal origin in lieu of soybean protein[1]	2nd or 3rd	79 (10)	25.3
4	S_{14}	IIa, VI	None	1st	75 (10)	74.7
	S_{14}	VI, V	None	2nd	87 (10)	89.6
	A_1[2]	..	Protein of animal origin in lieu of soybean protein[1]	3rd	84 (10)	7.2
5	S_{13}	Ib	None	1st	86 (10)	80.2
	S_{13}	Ib	None	2nd	98 (10)	97.9
	S_{13}	Ib	None	3rd	25 (4)	100.0
	S_{14}	Ib	Orotic acid, 100 mg per kg[1]	3rd or 4th	75 (10)	56.0

[1] Change of ration made after loss or weaning of preceding litter.
[2] Wilson Protein B replaced all of the purified soybean protein.
[3] Number of litters in parentheses.
[4] Young of 4 aborted litters not included.

after their first litters had incurred a mortality of 68.4% failed to have a curative effect. In fact, the second litters showed the increased mortality of the young which has been consistently observed when the diet contained the purified soybean proteins preparation Ia.

Since the rat can use folic acid as a precursor of the *Leuconostoc citrovorum* factor (CF; Sauberlich, '49), it was of interest in connection with these experiments to obtain some information concerning the urinary excretion of CF by rats as affected by the addition of folic acid or grass juice concentrate to the basal ration S_{13}. Immediately after the loss of a litter the urine was collected over a period of 4 days from representative rats described in tables 1 and 2 (group 1), respectively. The urine was frozen continuously during its collection and stored in the presence of toluene at — 15°C. until it could be analyzed.[6] The results of these analyses indicated that the mean daily citrovorum factor activity excreted in the urine was about 54, 1,460, and 267×10^{-3} μg, respectively, for animals fed ration S_{13} (4 rats), S_{13} plus 4.8 mg folic acid per kilogram (three rats) and S_{13} plus 10% grass juice concentrate (three rats). The grass juice contained $1{,}065 \times 10^{-3}$ μg CF activity per gram. These data in conjunction with those given in table 2 (group 1) demonstrate that the lactation failure cannot be ascribed to a deficiency of folic acid or of the citrovorum factor or to the inability of the animals to convert the former into the latter.

Evidence reported in the preceding paper indicated that the factors responsible for the prevention or cure of the syndrome under study are closely associated with some protein preparations of plant origin. To study the effect of "vitamin-free" casein on the lactation failure of rats, a group of animals that had previously been fed ration S_{13} were, after the loss or weaning of the second consecutive lit-

[6] The analyses were carried out through the courtesy of Dr. C. A. Baumann in the laboratories of the Department of Biochemistry of the University of Wisconsin by the procedures described by Sauberlich and Baumann ('48).

ter, transferred to ration C_2 in which the protein was supplied by "vitamin-free" casein. The data summarized in table 2 (group 2) show that after this curative change of the protein in the diet the mortality of the young decreased from 96% to about 36%. In prophylactic experiments in which three generations of rats were maintained on ration C_2, the mortality among 563 young born in 83 litters was 33.9%. It is evident, therefore, that "vitamin-free" casein contains one or more factors which can prevent or cure the lactation failure which is observed in a high incidence with diets containing purified soybean proteins. The incidence of abortions and fetal resorptions from 115 pregnancies on ration C_2 was about 14%, whereas with rations S_{13} or S_{14} it did not exceed 2%. This is pointed out because the three rations differed only with respect to the protein, the added methionine and orotic acid, respectively. It was also observed that the animals maintained on ration C_2 never presented the same sleek appearance at maturity and during the reproductive phase which was characteristic of rats maintained on rations S_{13} or S_{14}.

The curative effect of a crude protein preparation of animal origin on lactation failure

As a result of the pronounced prophylatic and curative effect of "vitamin-free" casein on the lactation failure of rats, it was of interest to investigate the effect on this syndrome of another protein of animal origin. For this purpose two groups of rats were used that had been fed rations S_{13} or S_{14}, respectively, since weaning and throughout the first two reproductive cycles. The parents of these rats in turn had been maintained on ration S_{13}. After loss or weaning of the second litter the rats in each group were transferred to ration A_1 in which the animal protein replaced the purified soybean protein. Table 2 (groups 3 and 4) summarizes the data on the mortality of the young observed in the litters born prior to the change of ration in each group, as well as

the first litter born after the change was made. It was evident that the complete substitution of the animal protein for the soybean proteins resulted in a decisive improvement of the lactation performance in both groups of rats. Further studies will have to determine whether the presence of orotic acid in ration S_{14} contributed to the very low mortality of about 7% among the young of rats subsequently transferred to A_1.

The curative and prophylactic effect of orotic acid on lacation failure

Experience with many dietary essentials for animals has shown that these compounds are also often required for the growth of some microorganisms. Orotic acid has been reported as a growth factor for a hemolytic streptococcus (Rogers, '44) for *Lactobacillus casei* (Chattaway, '44) and for *Lactobacillus bulgaricus* 09 (McRorie and Williams, '51; Wieland et al., '50; Wright et al., '50). It appeared worthwhile, therefore, to test the effect of orotic acid on the lactation failure of rats, particularly in view of the fact that this compound has been isolated from milk products (Biscaro and Belloni, '04; Wieland et al., '50; Huff et al., '50) and from fresh cow's milk (Hallanger et al., '53). Orotic acid has been previously considered for its possible function as a galactogogue by Sica and Cerecedo ('47) and found to be ineffective. The diet which they used contained purified casein but no added inositol, para-aminobenzoic acid or vitamin B_{12} and therefore differed markedly from the deficient rations used in the present experiments.

The curative effect of orotic acid on lactation failure was studied with a group of 10 rats which had been raised and maintained for two reproductive cycles on ration S_{13} containing the purified soybean protein preparation Ib. As shown in table 2 (group 5), there was again the progressively increasing, very high mortality of the young in the first two litters. As a further control, 4 of these animals were main-

tained during the third reproductive cycle on the same ration and their young then had a mortality of 100%. After the loss of the second or third litter, these rats were transferred to ration S_{14}, which differed from ration S_{13} only by the addition of 100 mg of orotic acid per kilogram. In the next litters, the pre-weaning mortality of the young decreased to 56%.

Such an increase in the survival of the young had never before been observed without a change in the diet and the possibility that it may have been the result of the administration of orotic acid clearly could not be overlooked. For this reason, prophylactic experiments were carried out in which litter-mate females whose mothers had been maintained throughout their lives on ration S_{13} were fed, respectively, rations S_{13} and S_{14} from weaning and throughout the production of from one to three litters. Unfortunately, the supply of protein preparation Ib which had been used for the curative experiments was exhausted by that time and the prophylactic experiments had to be carried out with other preparations that became available. The results summarized in table 3 fail to disclose a beneficial effect of the prophylactic feeding of orotic acid on the lactation performance of the rat. This was confirmed in a second prophylactic experiment

TABLE 3

Prophylactic effect of orotic acid on mortality of young

(Litter-mate mothers fed rations S_{13} and S_{14}[1] since weaning. Protein preparations IIa, V, IV, IX, X)

RATION OF MOTHERS	LITTER SEQUENCE	NUMBER OF YOUNG BORN	MORTALITY IN 21 DAYS
			%
S_{13}	1st	149 (20)[2]	60.4
S_{14}[1]	1st	134 (18)	41.7
S_{13}	2nd	147 (17)	62.5
S_{14}[1]	2nd	131 (15)	72.4
S_{13}	3rd	113 (13)	67.3
S_{14}[1]	3rd	107 (12)	68.3

[1] Ration S_{14} contained 100 mg of orotic acid per kilogram.

[2] Number of litters in parentheses.

involving a total of 83 litters from litter-mate mothers, raised and maintained on rations S_{13} and S_{14}, respectively. It has been pointed out in the preceding paper that the adequacy of various preparations of purified soybean protein for the support of lactation is subject to variations for reasons which are at present unknown. Therefore, the results of the curative and prophylactic experiments with orotic acid are not strictly comparable. It appears evident, however, from the results presented in table 3 that if orotic acid is involved in the deficiency syndrome under study, it is not the only factor concerned and probably not a major one. In view of the evidence presented in table 2, however, it would be premature to dismiss orotic acid from further consideration in these studies.

TABLE 4

Effect of liver concentrate added to the rations of mother rats fed diets S_{13} or S_{14} on the mortality of their young [1]

	LITTER MATES	
	Injected	Uninjected
Number of rats used	29	33
Mortality in 21 days, %	89.5	84.8

[1] Subcutaneous injection of 10 mg beef liver paste, Cohn Fraction D, into young shortly after birth.

Miscellaneous pertinent observations

As is shown in table 4, the subcutaneous injection, shortly after birth, of 10 mg of a beef liver paste [7] into the young of mothers maintained on the deficient diets failed to decrease the mortality of the injected animals. This observation can be interpreted as further evidence that the deficiency syndrome under study is primarily a lactation failure and that the liver concentrate used could not be substituted for compounds in which the mothers' milk was deficient. Rats maintained on ration S_{13} retained the ability to synthesize ascorbic acid. This has been demonstrated by the isolation

[7] Cohn Fraction D, Abbott Laboratories.

through chromatographic procedures (Probst and Schultze, '50) of the pure 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid from the urine of these rats.

DISCUSSION

The evidence presented in this and the preceding paper that there is a gradual development of lactation failure in rats maintained on diets containing purified soybean proteins, and that this condition can be prevented or cured by dietary means, raises a question concerning the nature of the factors involved. Until these factors have been recognized by isolation or other means, their identity is a matter of conjecture. The available evidence, however, permits some consideration of these factors in relation to various dietary essentials required by the rat and in relation to observations of other investigators dealing with dietary requirements for the lactation of the rat. For the purpose of this discussion and as a guide for further studies, it appears reasonable to consider that the lactation failure described may be affected by more than one factor, in the sense that two or more compounds of entirely different structure or several compounds of closely related structure may have preventive activity. The gradual development of the deficiency syndrome, coupled with the fact that we have not succeeded in producing a 100% incidence of lactation failure in large groups of rats, suggests that the basal ration S_{13} is not completely deficient or that its effects may be modified to a variable degree by the activity of the intestinal microflora. The available evidence, however, indicates that the lactation failure is not the direct result of a deficiency of one or more of the recognized water-soluble or fat-soluble vitamins or of amino acids because:

1. The basal ration S_{13} contained what may be considered liberal amounts of vitamins A, D, E, K, of thiamine, riboflavin, pyridoxine, pantothenic acid, nicotinic acid, biotin, vitamin B_{12}, choline, inositol, and para-aminobenzoic acid as well as some folic acid, in addition to unsaturated fatty acids, a

salt mixture and digestible proteins which by analysis were shown to furnish sufficient quantities of the essential amino acids. Increased quantities of folic acid failed to support better lactation.

2. The purification of the soybean proteins by aqueous and alcoholic extraction should remove all but traces of the recognized water-soluble and fat-soluble vitamins. In spite of this, one of the protein preparations (preparation VIII a, table 4, Schultze, '53) had a pronounced prophylactic and curative effect on the lactation failure. (Another preparation with prophylactic activity has since been encountered.)

3. The addition to the diet of a grass juice concentrate which contained considerable quantities of water-soluble vitamins and of ash failed to have a curative effect.

4. "Vitamin-free" casein, which is relatively low in recognized water-soluble and fat-soluble vitamins, had a pronounced curative and prophylactic effect.

5. The deficient rats retained the ability to synthesize ascorbic acid.

Keane et al. ('51) have presented evidence for the existence "in wheat germ oil and other natural fats (corn oil)" of an unidentified factor which is necessary to permit the female rat to rear her young. The deficient ration with which they demonstrated this factor contained 24% of "vitamin-free" casein.[8] The same type of casein, however, had a marked curative and prophylactic effect on the lactation failure described in this paper. In addition, the deficient ration used for these studies contained 1% of corn oil and 7% of a partially hydrogenated vegetable fat. Therefore, it appears to be extremely unlikely that the lactation failure studied in the two laboratories is due to the same dietary deficiency. The deficient diet with which El Ridi et al. ('50) detected the existence of a fat-soluble lactation factor in Fenu Grek oil contained 8% of casein.

Nakahara and his associates have published extensively concerning the existence of two lactation factors, vitamin L_1

[8] See footnote 2, page 246.

and vitamin L_2, required by the rat which, according to their recent publications, can be replaced by anthranilic acid (Nakahara et al., '45) and adenylthiomethyl pentose (Nakahara et al., '43), respectively. These observations remain to be confirmed and in view of the great difference in the composition of the rations used by Nakahara et al. and those employed in this study, no valid conclusions can be drawn at present concerning the identity of the L vitamins and the factors concerned with lactation failure discussed in this paper. It is possible that the deficiency syndrome described here is related to the observations of Piccioni et al. ('51), who observed a high mortality of young rats born to mothers fed a diet containing cereal grains, exhaustively washed casein, fresh vegetables and yeast.

Many earlier studies which, through the use of rations containing purified casein or ingredients of plant origin, indicated the existence of unrecognized dietary factors for the reproduction and lactation of the rat were no doubt complicated by a deficiency of vitamin B_{12}. Since vitamin B_{12} was a component of the rations used in the present study, and since it has been shown to exert a great effect on the preweaning mortality of rats (Schultze et al., '52; Nell and Phillips, '50), comparison of the results of this with those of earlier studies would be largely speculative. The present results, however, confirm many earlier observations that the stress of lactation imposes special dietary requirements on the maternal organism which are otherwise not evident and which for the rat are not clearly defined at present.

SUMMARY

Experiments on the effect of various dietary supplements on a lactation failure in rats induced by the feeding of diets containing purified soybean proteins have indicated the following:

1. This condition could not be cured by the feeding of increased amounts of folic acid or by the addition to the maternal diet of as much as 10% of a grass juice concentrate.

2. "Vitamin-free" casein produced a marked curative and prophylactic effect.

3. A strong curative effect was obtained by the feeding of a crude animal protein prepared from defatted brain and spinal cord.

4. Orotic acid appeared to have a curative effect in one experiment. In prophylactic experiments, however, no consistent benefit from orotic acid could be demonstrated.

5. Rats maintained on the deficient diets retained the ability to convert folic acid to the *Leuconostoc citrovorum* factor and to synthesize ascorbic acid.

6. It appears that the dietary factors responsible for the prevention or cure of this lactation failure are different from the recognized fat-soluble and water-soluble vitamins. Their relation to other factors concerned with the maintenance of lactation in the rat has been discussed.

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THE INFLUENCE OF VARIOUS CARBOHYDRATES ON THE UTILIZATION OF LOW PROTEIN RATIONS BY THE WHITE RAT [1]

I. COMPARISON OF SUCROSE AND CORNSTARCH IN 9% CASEIN RATIONS

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INTRODUCTION

Various investigators have demonstrated that under certain experimental conditions both dextrin and cornstarch support better growth of rats and chicks than does sucrose. The majority of the reports are of studies of B vitamin deficiencies in which it has been shown that dextrin promotes greater intestinal synthesis of a number of the B vitamins than does sucrose (Elvehjem, '48). There remain other experiments, in particular those in which 9% casein rations have been used with different carbohydrates for the study of either niacin-tryptophan or methionine deficiencies in rats, in which the growth differences observed cannot be explained satisfactorily on the basis of increased intestinal synthesis of B vitamins.

During some routine feeding experiments Harper and Lepp [2] observed that chicks which were fed sucrose, in a ration containing 18% casein that was considered to be com-

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[2] Unpublished results. University of Wisconsin, 1947.

plete, grew at a somewhat slower rate than those receiving dextrin as the source of carbohydrate. The small growth differences were reduced when either the level of casein or the level of methionine in the sucrose ration was increased. These experiments were performed before the discovery of vitamin B_{12} and before much of the later work on chick growth factors. Recently, however, Monson, Dietrich and Elvehjem ('50) have reported marked growth differences between chicks receiving dextrin and those receiving sucrose which could not be eliminated by the addition of various supplements to the sucrose ration.

During investigations of the relationship between niacin and tryptophan several Wisconsin workers (Krehl et al., '46; Henderson et al., '47; Hankes et al., '48; Lyman and Elvehjem, '51) have reported substantial differences in growth between groups of rats fed 9% casein rations containing sucrose and groups receiving similar rations with dextrin as the source of carbohydrate. That the growth differences were not caused by a niacin deficiency can be seen from the results of Krehl et al. ('46), which show that although the addition of niacin to the sucrose ration produced a slight improvement, and the addition of tryptophan produced a greater improvement, in neither case was the growth of rats receiving the sucrose diet equal to that of animals fed dextrin.

While studying a methionine deficiency produced by feeding rats 9% casein as the entire source of protein, Hall and Sydenstricker ('46) observed that animals receiving sucrose grew very slowly even when 0.5% methionine was included in the ration. However, when sucrose was replaced by cornstarch, and inositol, nicotinic acid and *p*-aminobenzoic acid were added, growth was equal to that obtained with an 18% casein ration. These workers reported that supplementation of the sucrose ration with 5 essential amino acids produced a similar growth response. Since their 18% casein ration supported an average growth rate of only 21 gm per week,

it appears that some factor was limiting even in their best ration.

The possibility of a common explanation for these observations seemed to merit further study. The investigation reported here consisted of a comparison of sucrose and cornstarch as dietary carbohydrates for rats fed various levels of casein and methionine.

EXPERIMENTAL

Young white rats of the Wistar strain weighing from 40 to 60 gm were divided into similar groups on the basis of sex and initial body weight. The animals were maintained in individual cages with raised screen floors and were fed ad libitum. Each animal was weighed weekly and individual food consumption records were kept during the experimental periods of 28 days.

The percentage composition of the basal ration was as follows: sucrose or cornstarch 81.2, casein 9, corn oil 5, salts (Harper et al., '52) 4, methionine 0.5, choline 0.2, inositol 0.1. Vitamins were added so as to provide, in milligrams per 100 gm of ration: thiamine 0.5, riboflavin 1.0, pyridoxine 0.5, niacin 0.5, calcium pantothenate 2.5, folic acid 0.02, biotin 0.02, *p*-aminobenzoic acid 30, α-tocopherol 2.5 and 2-methyl-1,4-naphthoquinone 0.2. Radiostoleum (B. D. H.) to provide 6,000 I.U. of vitamin A and 1,200 I.U. of vitamin D per 100 gm of ration was mixed with the corn oil. The various experimental rations are listed with the growth data in table 1. Supplements were added, or alterations in the levels of casein and methionine were made, at the expense of carbohydrate.

The standard error of the mean weekly gain in weight and of the mean daily food intake for each group is reported. Since most groups contained both males and females, and since the females usually grew at a slower rate than the males, the standard errors are generally larger than would be obtained for groups consisting of rats of a single sex (cf. experiment 7, table 1). In order to test statistically the

TABLE 1

Effect of various rations containing either sucrose or cornstarch on growth, food intake and food utilization of rats [1]

EXPT. NO.	GROUP NO.	CASEIN	METHIONINE	EXPERIMENTAL RATION Carbohydrate	SUPPLEMENT	WEEKLY GAIN [2]	DAILY FOOD INTAKE [2]	GAIN/100 GM RATION INGESTED
		%	%			gm	gm	gm
1	1	18	0.5	Sucrose	None	34.8 ± 3.4	12.2 ± 0.7	40.6
	2	18	0.5	Cornstarch	None	38.0 ± 3.6	13.0 ± 0.6	41.6
	3	9	0.5	Sucrose	None	14.7 ± 2.8	9.0 ± 0.8	23.2
	4	9	0.5	Cornstarch	None	24.8 ± 2.9	11.8 ± 0.6	30.0
2	5	18	0.5	Sucrose	None	28.9 ± 2.6	11.0 ± 0.5	37.6
	6	9	0.5	Sucrose	None	10.8 ± 1.4	7.5 ± 0.6	20.6
	7	9	0.5	Sucrose	0.4% liver extract	14.6 ± 1.2	8.5 ± 0.3	24.3
	8	9	0.5	Sucrose	double B vitamins	15.1 ± 0.6	8.5 ± 0.2	25.1
	9	9	0.5	Cornstarch	None	24.5 ± 2.4	11.5 ± 0.5	30.4
	10	9	0.5	Cornstarch	0.4% liver extract	23.1 ± 2.9	11.1 ± 0.8	29.8
3	11	18	0.5	Sucrose	None	33.5 ± 3.9	12.5 ± 0.7	38.2
	12	9	0.5	Sucrose	None	13.0 ± 0.9	8.1 ± 0.3	23.0
	13	9	0.5	Sucrose	Essential amino acids [3]	24.8 ± 3.3	10.1 ± 0.8	34.9
	14	9	0.5	Sucrose	Non-essential a.a.[3]	11.4 ± 2.1	7.6 ± 0.5	21.6
	15	9	0.5	Cornstarch	None	25.7 ± 4.2	12.0 ± 1.1	30.8
	16	9	0.5	Cornstarch	Non-essential a.a.[3]	28.2 ± 2.2	12.6 ± 0.6	32.1
4	17	9	0.5	Sucrose		14.0 ± 0.9	9.1 ± 0.3	21.9
	18	9	0.5	Sucrose	1% sulfasuxidine	15.2 ± 1.1	9.0 ± 0.4	23.7
	19	9	0.5	Cornstarch		22.3 ± 2.5	11.0 ± 0.4	29.0
	20	9	0.5	Cornstarch	1% sulfasuxidine	28.6 ± 2.2	12.6 ± 0.9	32.4
5	21	11	0.5	Sucrose	None	20.0 ± 1.1	9.2 ± 0.3	31.2

	20	9	0.5	Cornstarch	1% sulfasuxidine	28.0 ± 2.2	12.0 ± 0.9	32.4
5	21	11	0.5	Sucrose	None	20.0 ± 1.1	9.2 ± 0.3	31.2
	22	13	0.5	Sucrose	None	28.9 ± 4.3	10.8 ± 0.8	38.2
	23	15	0.5	Sucrose	None	32.7 ± 3.3	12.2 ± 0.7	38.3
	24	11	0.5	Cornstarch	None	31.0 ± 3.4	12.3 ± 0.7	36.0
	25	13	0.5	Cornstarch	None	32.4 ± 3.2	12.4 ± 0.6	37.6
	26	15	0.5	Cornstarch	None	34.1 ± 2.8	12.9 ± 0.4	37.6
6	27	9	..	Sucrose	None	6.0 ± 1.0	5.7 ± 0.3	15.1
	28	9	0.05	Sucrose	None	8.5 ± 1.2	6.4 ± 0.3	19.1
	29	9	0.10	Sucrose	None	10.8 ± 1.2	7.0 ± 0.3	22.0
	30	9	0.50	Sucrose	None	13.0 ± 0.5	7.7 ± 0.5	24.0
	31	9	0.50	Sucrose	1% bacitracin-B_{12} conc.	11.0 ± 0.4	7.1 ± 0.4	22.1
	32	9	...	Cornstarch	None	16.1 ± 1.2	9.0 ± 0.5	25.5
	33	9	0.05	Cornstarch	None	18.0 ± 1.8	9.4 ± 0.5	27.3
	34	9	0.10	Cornstarch	None	17.2 ± 1.0	9.4 ± 0.4	26.4
	35	9	0.50	Cornstarch	None	25.1 ± 2.1	11.4 ± 0.5	31.3
	36	9	0.50	Cornstarch	1% bacitracin-B_{12} conc.	23.5 ± 2.8	10.9 ± 0.8	30.8
7	37	9	0.5	Sucrose		12.2 ± 0.7	6.8 ± 0.4	25.8
	38	9	0.5	Sucrose	3% cellulose 2% agar	15.0 ± 0.7	7.7 ± 0.3	27.4
	39	9	0.5	Cornstarch		25.8 ± 1.2	10.9 ± 0.4	33.8
	40	9	0.5	"Cornstarch-dextrin"		25.0 ± 0.7	11.2 ± 0.3	32.0
	41	9	0.5	"Potato starch-dextrin"		22.0 ± 0.9	10.3 ± 0.2	30.1

[1] Groups consisted of three male and three female rats except for experiment 1 in which 4 males and two females were used, experiment 7 in which 6 males were used and groups 30, 31, 35 and 36, which consisted of three males and two females.

[2] Mean ± standard error of the mean.

[3] The amino acid supplements are described in the text.

significance of differences which were in doubt, and in order to eliminate, for such comparisons, the variation noted between males and females within a single group, the groups were paired on the basis of sex and initial body weight; i.e., on the basis on which the groups had originally been set up. Values determined for 't' and the corresponding values at P = 1% or 5% (Goulden, '39) are presented in the text below.

RESULTS

The first experiment (table 1) consisted of a comparison of cornstarch and sucrose in both 9% and 18% casein rations. With 18% casein rations, no marked growth differences could be demonstrated between groups of rats fed either of these carbohydrates. The male rats in these two groups gained at the rate of 40 to 44 gm per week (double the rate reported by Hall and Sydenstricker, '46), which indicated that the basal ration was adequate in all respects when the protein level was increased. The results for the 9% casein rations show clearly that when a suboptimum amount of casein was fed the rate of growth was much slower with sucrose than with dextrin. The difference between these two groups (three and 4) is statistically highly significant (t = 8.11, t at 1% = 4.03). In the two succeeding experiments the groups which were fed 9% casein and sucrose were considered as negative controls and those fed 9% casein and cornstarch as positive controls.

The second experiment was designed to determine whether increased amounts of B vitamins or the addition of a liver extract [3] might improve the sucrose ration. A slight growth response over that of the negative controls, group 6, was obtained with both the liver extract and the double amount of B vitamins. The differences here are just statistically significant (between groups 6 and 7, t = 2.82, t at 5% = 2.57; between groups 6 and 8, t = 3.21, t at 5% = 2.57). Although the rate of growth of the negative controls in this experi-

[3] Wilson's.

ment was less than average, no statistically significant differences could be demonstrated between this group and the other 4 comparable negative control groups from the other experiments; furthermore, no significant differences could be demonstrated among groups 7 and 8 and the other negative controls. It seems doubtful, therefore, whether the increases obtained are beyond the range of experimental error. Certainly the sucrose ration, supplemented with either increased amounts of B vitamins or with the liver extract, did not support a rate of growth which approached that of the positive controls, group 9, which received cornstarch.

The results of experiment 3 indicate the effects of supplements of certain amino acids. The supplement of essential amino acids provided 0.4% lysine, 0.15% valine, 0.2% threonine, 0.1% tryptophan and 0.2% histidine in the ration. These amino acids were considered to be limiting in rations containing only 9% casein and were included at the levels used by Hall and Sydenstricker ('46). The supplement of non-essential amino acids provided 0.5% glycine and 0.55% glutamic acid in the ration. It is apparent from the growth data that neither the sucrose nor the cornstarch ration was improved by the addition of non-essential amino acids; however, rats receiving the sucrose ration supplemented with *essential amino acids* grew at almost the same rate as those receiving the cornstarch ration.

In order to determine whether the inclusion of succinylsulfathiazole in either of the basal rations might affect the rate of growth, experiment 4 was carried out. No growth reductions resulted; in fact, if anything a slight growth stimulation was obtained in both cases.

Experiment 5 was designed to determine whether growth differences, similar to those obtained between groups of rats fed 9% casein rations containing sucrose and those fed similar rations containing cornstarch, might occur when other levels of casein were fed. From table 2 it can be seen that the average rate of growth for the animals on the sucrose basal diet was 12.8 gm/week and for those receiving the

cornstarch basal diet it was 24.0 gm/week. When the casein in the sucrose ration was increased to 11%, the rate of growth of the animals receiving this ration, group 21, was only slightly less than that of the animals receiving the 9% casein-cornstarch ration. However, those receiving 11% casein and cornstarch, group 24, grew considerably better than groups receiving either 9% casein and cornstarch or 11% casein and sucrose. When the casein in the sucrose ration was increased to 13%, the rats receiving this ration, group 22, grew as well as those receiving the 11% casein-cornstarch ration. No

TABLE 2

Comparison of rate of growth, food intake, and food efficiency of rats fed rations containing 9% casein and 0.5% methionine with either sucrose or cornstarch

		NUMBER OF ANIMALS	AVERAGE 4-WK. GAIN	AVERAGE 4-WK. FOOD INTAKE	AVERAGE GAIN/INTAKE
			gm	*gm*	*gm/100 gm*
Sucrose	Males	16	52	227	22.9
	Females	13	50	226	22.2
	Total	29	51	226	22.6
Cornstarch	Males	16	105	334	31.3
	Females	13	85	295	28.8
	Total	29	96	317	30.3

marked growth differences were observed when the level of casein in either of the basal rations was raised beyond these percentages.

The influence of various levels of methionine and of a bacitracin-vitamin B_{12} supplement on the growth of rats fed either sucrose or cornstarch is shown by the results of experiment 6. With one exception (0.1% methionine with cornstarch, in which two of the male rats failed to gain normally during the last two weeks of the experiment) gradual increases in the rates of growth with increasing amounts of methionine in the rations were obtained with both carbohydrates. Nevertheless it can be seen from the results that groups receiving 9% casein, 0.5% methionine and sucrose

did not grow as well as those receiving only 9% casein and cornstarch without additional methionine.

The bacitracin-vitamin B_{12} supplement did not produce a growth response when fed with either carbohydrate.

The effects of some other carbohydrates on the growth of rats fed the 9% casein basal ration and the effect of including bulk in the sucrose basal ration were determined. From the growth data of experiment 7 it is apparent that "dextrin" (prepared from either cornstarch or potato starch by heating the moist starch for two hours in an autoclave at 121°C.) supports growth equivalent to that obtained with cornstarch. The inclusion of bulk in the sucrose ration was found to induce a rate of growth only slightly greater than that which occurred with sucrose alone.

The values presented in table 2 were obtained by averaging the results obtained with all the negative and all the positive control groups. The difference between the rate of growth of rats fed the sucrose basal ration and that of those fed the cornstarch basal ration is again emphasized. Also apparent from this table are the increase in food intake and the more efficient food utilization of animals receiving the cornstarch ration.

No marked differences in food utilization were found between the males and the females of groups receiving the sucrose ration. There was, however, slightly better utilization of the cornstarch ration by the males These observations are interesting in view of a report by Jones ('51) stating that females gain more rapidly than males on diets inadequate in protein. With 9% casein rations (carbohydrate unspecified) he obtained equal growth of males and females. While this was true with the 9% casein-sucrose rations used in this study, the better growth of males on the 9% casein-cornstarch ration would indicate the superiority of this ration.

DISCUSSION

Growth data from the results of several investigators who used similar rations were selected for comparison with the

values obtained in this study and are presented in table 3. With the exception of the values of Hall and Sydenstricker ('46), which are low, the data obtained from the literature differ only slightly from those presented in this paper. There is thus considerable evidence to support the observation that rats receiving low casein rations grow better with "dextrin" or cornstarch than with sucrose as the source of carbohydrate.

TABLE 3

Weight gains of rats fed sucrose, dextrin or cornstarch rations, as reported by several investigators

PROTEIN	CARBOHYDRATE	4-WK. WT. GAIN		GAIN/RATION INGESTED
		gm		*gm/100 gm*
20% casein	Sucrose	84 [1]		
18% casein + 0.5% methionine	Sucrose		139 [5]	
9% casein + 0.2% cystine	Dextrin	98 [2]		
9% casein + 0.5% methionine	Cornstarch	87 [1]	105 [5]	
9% casein + 0.2% cystine	Sucrose	55 [2]		
9% casein + 0.5% methionine	Sucrose	25 [1]	52 [5]	22.6 [5]
8% casein + 0.4% methionine	Sucrose	48 [3]		
7.5% casein + 0.6% methionine	Sucrose	51 [4]		21.4 [4]
9% casein	Sucrose		24 [5]	
8% casein	Sucrose	28 [3]		

[1] Hall and Sydenstricker ('46).
[2] Lyman and Elvehjem ('51).
[3] Reisen et al. ('46).
[4] Lewis and Fajans ('51).
[5] Harper and Katayama, present paper.

Many explanations for these growth differences might be offered, but from the evidence available at present no single explanation can be proposed.

From the data presented in tables 1 and 2 it is evident that rats receiving the cornstarch basal ration ate much more than those receiving sucrose and it is tempting to suggest that palatability is important in altering the food intake and consequently the rate of growth. Hegsted and Haffenreffer ('49) have suggested that food intake is maintained at a relatively constant percentage above the basal requirement and

that if the diet is adequate the animal grows well and its food intake increases accordingly; however, if the ration is poor, both growth and food intake are less. The values obtained for gain per 100 gm of ration consumed (table 2) indicate the superiority of the cornstarch ration. This, together with the increase in food intake observed when small amounts of essential amino acids were added to the sucrose ration, and when the level of casein was increased, invalidate the hypothesis that palatability is significant.

It is well known that most starches contain small amounts of protein and it might be suggested that the low casein ration is supplemented by the amino acids from the protein in the starch. In the present study the amount of nitrogen in the cornstarch, as determined by the Kjeldahl method, was equivalent to only 0.37% protein; for the "dextrin" from potato starch the value was 0.14%. Since the addition of 2% casein to the sucrose ration was required to produce growth approaching that obtained with cornstarch, and since corn gluten is inferior to casein as a source of essential amino acids, it is improbable that the amount of protein in the cornstarch is significant. Furthermore, the two "dextrin" preparations, which differed greatly in protein content, supported approximately the same rate of growth.

Monson et al. ('50) have suggested that an unknown growth factor may be required by chicks fed sucrose rations and have suggested that this may be synthesized by intestinal microorganisms when dextrin is fed, or may be present in the dextrin. In this study rats fed the sucrose ration supplemented with 5 essential amino acids grew as well as those fed dextrin; therefore it is unlikely that such an unknown growth factor is important for the rat.

The marked growth responses obtained when the sucrose ration was supplemented with 5 essential amino acids or when the level of casein was increased would definitely indicate that a greater quantity of essential amino acids was available for growth when cornstarch was used as the source of carbohydrate in place of sucrose.

Hall and Sydenstricker ('46) suggested that essential amino acids may be synthesized by the intestinal microorganisms of rats fed cornstarch. It is possible that microorganisms in the intestinal tract of the rat, like those in the rumen of ruminants, synthesize amino acids essential for mammals and that small amounts of these amino acids may be made available through the hydrolysis of dead bacterial cells. It is doubtful, however, whether much hydrolysis would occur high enough in the digestive tract for absorption to take place. If intestinal synthesis is important, microorganisms which are not affected by succinylsulfathiazole must be responsible, because no growth depression was obtained when 1% of this compound was included in the cornstarch ration.

The converse explanation — that the intestinal flora of rats fed sucrose use greater quantities of amino acids than the intestinal flora of animals fed "dextrin" or cornstarch — has not been previously suggested but would seem to be equally tenable on the basis of the evidence available at present.

Another hypothesis, suggested by Krehl et al. ('46) and by Lyman and Elvehjem ('51) to explain the apparently lower tryptophan requirement of rats receiving dextrin rations which were deficient in tryptophan, is that the slower passage of the dextrin ration through the digestive tract and its larger bulk permit slower digestion and more economical utilization of the other dietary constituents.

Harper, Katayama and Jelinek ('52) have shown that casein is not completely digested by rats when raw potato starch is provided as the dietary carbohydrate. Thus at least one carbohydrate can cause a reduced rate of digestion of protein. Casein was almost completely digested when either cornstarch or sucrose was fed, so no information regarding differences between the effects of these two carbohydrates was obtained. Monson et al. ('50) have shown that dextrin rations pass through the gastrointestinal tract of the chick more slowly than do sucrose rations. It is thus possible that the rate of digestion may be slower when rations containing

cornstarch or "dextrin" in place of sucrose are fed and that this may permit more efficient utilization of protein.

Munro ('51), in a recent review of the relationship between carbohydrate and protein in nutrition and metabolism, cited several studies in which the growth of rats was reduced when protein and carbohydrate were fed separately. It may be that sucrose is removed more rapidly from the digestive tract than is cornstarch, and that glucose and amino acids are absorbed together for a longer period of time when cornstarch is fed, and that this permits better utilization of the protein.

The possibility that the more efficient utilization of casein and methionine by rats fed rations containing cornstarch or "dextrin" in place of sucrose is due to the interaction of several factors cannot be overlooked. Regardless of the final explanation of the observations reported here, one feature of the results might be emphasized. If, as is indicated, good growth of rats can be obtained with a lower level of casein when dextrin is substituted for sucrose, then it follows that the type of carbohydrate in the ration may, at least under certain experimental conditions, when intact casein is used as a source of amino acids, modify the requirement of the rat for amino acids.

SUMMARY

The influence of sucrose and that of cornstarch on the utilization by the white rat of low casein rations supplemented with methionine have been determined. Cornstarch supported better growth of rats receiving 9% casein rations than did sucrose. The growth differences were eliminated by increasing either the level of casein or the levels of 5 essential amino acids in the sucrose ration but were unchanged when liver extract was included, when the quantities of the B vitamins were increased or when certain non-essential amino acids were included.

The growth of rats fed low casein rations deficient in methionine was also shown to be better when cornstarch was substituted for sucrose as the source of carbohydrate.

Various explanations for these observations have been discussed. The growth differences observed cannot be explained satisfactorily by differences in palatability of the rations, by the influence of protein present in cornstarch or by a deficiency of an unknown growth factor. They are apparently due to the availability of greater quantities of certain essential amino acids when sucrose is replaced by cornstarch. It seems improbable that intestinal microorganisms play a significant role in this regard and it is deemed more likely that cornstarch, by reducing the rate of passage of the ration through the gastrointestinal tract, promotes more efficient utilization of low casein rations.

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UTILIZATION OF AMINO ACIDS FROM FOODS BY THE RAT

I. METHODS OF TESTING FOR LYSINE [1]

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ONE FIGURE

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In addition to information on the amounts of individual amino acids contained in foods and on the amounts required by the animal for optimum nutrition, studies on the actual amounts utilized by the animal are also needed for a definitive nutritional evaluation of protein quality. Considerable attention has been directed toward evaluating the effect of processing on the rate and extent of release of amino acids *in vitro* by proteolytic enzymes (Riesen et al., '47; Hankes et al., '48; Beuk et al., '49; Clandinin et al., '51), on the digestibility or availability of specific amino acids in foods (Pearce et al., '47; Kuiken and Lyman, '48; Kuiken, '52), and on the ability of the test product fed at a limited level of intake to support growth of the rat or chick or weight recovery of the protein-depleted rat (Block et al., '46; Frost and Sandy, '48; Beuk et al., '49; Evans and McGinnis, '46; Wissler et al., '47; Mitchell et al., '49; McBride et al., '51). Although these

[1] We are indebted to E. I. DuPont de Nemours and Company for supplying the L-lysine hydrochloride, DL-lysine hydrochloride and DL-methionine; to the Dow Chemical Company for the DL-tryptophan; to Merck and Company for the vitamin B_{12}; to the Lederle Laboratories Division, American Cyanamid Company, for the pteroylglutamic acid; and to the Pacific Vegetable Oil Corporation for the sesame meal used in these studies.

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studies are of great value, they do not provide information on the utilization of any of the amino acids from foods that are not limiting with respect to the growth of the animal. It is of importance, therefore, to extend the nutritional evaluation of proteins to a study of the amounts of individual amino acids which are utilized from a food to support a specific function, or specific functions, of the test animal (i.e., weight gain, hematopoiesis and so forth). In an earlier study (Schweigert, '48), the percentage of tryptophan utilized from several foods to support the growth of chicks was found to range from 20 to 65 for different test products (tryptophan was the only limiting nutrient in the test diet).

In the present work, the possibility of using the protein-depleted rat as a test animal for studying the quantitative utilization of amino acids from foods was investigated. The initial studies have been conducted with lysine, and experiments on the type of basal ration used, the repeatability of the assays and other criteria of reliability have been carried out. A preliminary report of this phase of the work has been published (Schweigert et al., '52). The application of these techniques to determine the percentage of lysine utilized from several foods is presented in the accompanying paper (Guthneck et al., '53).

EXPERIMENTAL AND RESULTS

Male rats (Holtzman) that averaged 250 gm in weight were available for this study. These rats had been fed a stock diet [2] for three weeks. The rats were then fed a purified ration without protein additions for a 12- to 15-day period in individual experiments, and the loss of body weight ranged from 20 to 25%. The basic composition of this diet is presented in table 1. A similar depletion diet was used by Frost and Sandy ('48). The animals were then randomized among the experimental groups according to weight and weight loss during depletion. Four to 6 rats were used in each group in the individual experiments. In the initial experiments the

[2] Rockland Rat Pellets Complete.

amino acid supplements in the basal ration used for measuring the lysine utilization were purified amino acids (table 2). In later studies the basal ration consisted of sesame meal plus certain amino acid supplements (table 2). These additions and graded levels of lysine, or foods that contained lysine, were made to the depletion ration at the expense of sucrose. The tests were conducted for an 11- or 12-day period. The animals were then fed the stock ration, followed by the protein depletion ration, and used for a second test in a similar

TABLE 1

Composition of depletion ration

INGREDIENTS	AMOUNT	INGREDIENTS	AMOUNT
	%		*mg/100 gm ration*
Salts [1]	4.0	Thiamine HCl	0.6
		Riboflavin	0.6
		Pyridoxine HCl	0.6
Corn oil	4.7	Ca pantothenate	4.0
		Nicotinic acid	2.0
Fish liver oil (3,000 A, 400 D/gm)	0.3	Choline chloride	100.0
		Inositol	100.0
		Pteroylglutamic acid	0.2
Sucrose	to 100	Biotin	0.01
		2-Methyl 1,4 naphthoquinone	3.0
		p-Aminobenzoic acid	30.0
		Vitamin B_{12}	0.002

[1] Hegsted et al. ('41).

manner. All animals were housed in individual cages with raised screen bottoms, and food and water were provided ad libitum. The lysine content of the foods tested was determined microbiologically with *Leuconostoc mesenteroides* P-60 as the test organism (Schweigert et al., '49).

The rate of gain of protein-depleted rats fed the amino acid ration plus excess lysine (700 mg/100 gm) was rapid and uniform. The total gain approximated 60 gm for the 12-day test period and continued at the same rate when the stock diet was fed. Tests with graded levels of lysine (fig. 1) showed

that a graded, essentially linear response occurred when the tests were conducted for 4, 8 or 11 days, and that the requirement for L-lysine approximated 450 mg/100 gm ration. Food efficiency data (grams gain/gram food consumed) were in good agreement with the growth data.

TABLE 2

Composition of amino acid supplements

AMINO ACID RATION [1]			SESAME MEAL RATION [2]		
Constituent	Form	mg/100 gm	Constituent	Form	amount/100 gm
Arginine	L-HCl	510	Sesame meal		19.2 gm
Histidine	L-HCl-H_2O	347	Diammonium citrate		3.2 gm
Isoleucine	DL	1330	Isoleucine	DL	130 mg
Leucine	L	1265	Leucine	L	10 mg
Methionine	DL	360	Methionine	DL	37.5 mg
Phenylalanine	DL	533	Histidine	L-HCl-H_2O	36 mg
Threonine	DL	800	Threonine	DL	50 mg
Tryptophan	DL	185	Valine	DL	20 mg
Valine	DL	1440			
Alanine	DL	577			
Aspartic acid	DL	648			
Cystine	L	334			
Glutamic acid	L	2450			
Tyrosine	L	660			
Glycine		51			

[1] The amounts used are based on those used by Wissler et al. ('48).

[2] The sesame meal contained 45.9% crude protein and 1.16% lysine. Diammonium citrate was added at a level to supply the same total nitrogen to the ration as that in the amino acid ration. The other amino acids were added at levels to provide a 40% excess of each amino acid over the estimated requirement, which was based on the report of Benditt et al. ('50) and the analyses reported by Grau and Almquist ('44).

A further comparison was made with rats receiving either the basal amino acid ration plus 300 mg lysine or the basal ration plus an increase of 50% in the amounts of all amino acids in the basal ration and 300 mg/100 gm of L-lysine (added as the L-HCl-H_2O). The rates of gain were almost identical with this limiting level of lysine for the two groups (54.5 and 53.7 gm, respectively). The extra nitrogen supplied by the additional amino acids exceeded that contributed by any

food product, including the cereals low in lysine. Thus, although additional nitrogen (protein) has been shown to increase the level of lysine required for maximum growth of the chick (Grau, '48), it did not affect the response to

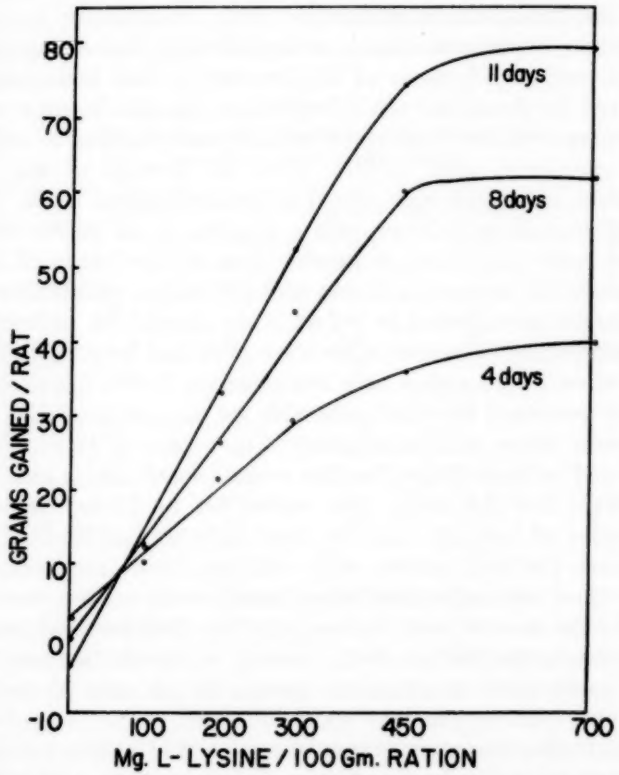

Fig. 1 Ratio of gain for groups of protein-depleted rats fed the amino acid basal ration plus graded lysine supplements for periods of 4, 8 or 11 days.

limiting levels of lysine for the protein-depleted rat. These findings indicated that the experimental design was satisfactory for testing the quantitative utilization of lysine from foods with the protein-depleted rat.

Several food products were selected and one or more levels added to the basal diet to provide known amounts of lysine (150 to 350 mg/100 gm; see fig. 1) based on microbiological analysis. On the basis of the rates of gain for groups fed graded levels of L-lysine, the percentage of lysine utilized from the foods was calculated.

Further experiments were conducted with the sesame meal ration, not only because of the expense of the amino acids required for the amino acid basal ration, but also because with the amino acid basal ration the animal was required to utilize only one amino acid (lysine) from the protein of the test products; all others were added as purified amino acids. The use of sesame meal to provide a portion or all of the other amino acids permitted a further test of the value of this approach for measuring amino acid utilization quantitatively, and might be expected to reflect more closely the utilization of lysine when the amino acids were provided from more than one source. The amino acid supplements (table 2 and footnotes) increased the total gain with the sesame meal diet and the assay range was satisfactory (i.e., a gain of 18 vs. 85 gm with and without lysine for the sesame meal ration and — 4 vs. 80 gm for the amino acid ration for an 11-day period). It is also of interest that the total gain was approximately the same for both rations with optimum lysine supplementation. That the amino acid supplements were needed in addition to the sesame meal and ammonium citrate was shown in tests conducted for an 8-day period in which the rats fed the amino acid supplements gained 67 gm and those not receiving the supplement gained 41 gm.

The food protein supplements were added at a lower level (to provide approximately 150 mg lysine per 100 gm of ration) with the sesame meal ration, since the sesame meal provided 223 mg lysine per 100 gm of ration.

The consistency of the quantitative results for lysine utilization by the rat after 4, 8 and 11 days on experiment is indicated in table 3. On an over-all basis, the percentage utilization of lysine was of greater uniformity with the use of the sesame

meal basal ration. In certain cases (i.e., soybean meal, beef-round and split peas) the utilization observed with the amino acid ration was somewhat lower for the first 4 days than for the later periods; therefore, the per cent lysine utilized was calculated from the data obtained from the 4th to the 11th days on experiment. The results for lysine utilization with the sesame meal ration were calculated for the 11- or 12-day period.

TABLE 3

Per cent of lysine utilized by the rat from different products at different experimental periods

PRODUCT TESTED	PER CENT LYSINE UTILIZED			
	4 Days	8 Days	11 Days	4–11 Days
Amino acid ration				
Casein	71	71	72	71
Beef round	41	69	66	84
Split peas	73	88	90	104
Soybean meal	66	79	80	89
Sesame meal ration				
Casein	75	81	81	
Dried eggs	95	106	98	
Fresh pork	105	90	96	
Luncheon meat	77	77	78	

It was also of importance to determine the per cent of the lysine ingested from different foods that were utilized when the amount of sample (lysine) fed to different groups was varied. Results for these tests are presented in table 4. The standard errors of the means for these data are also indicated (see also table 2 of the accompanying paper). It will be noted that good agreement in the results was obtained in all cases, except for uncooked beef rib. No trends of lower or higher utilization of lysine were evident when the sample size was increased. Tests with DL-lysine showed that the D-isomer was not utilized.

A further evaluation of the results was afforded by determinations of the lysine utilization on repeat experiments with

the same animals and by the use of both basal rations with the same food products (table 5). As in previous comparisons, most of the results were in good agreement. The results were not consistently higher or lower with either the amino acid or the sesame meal basal ration. The agreement in the data for soybean oil meal and for split peas with the two basal rations

TABLE 4

Per cent lysine utilized when different levels of test product are fed

PRODUCT TESTED	AMOUNT (dry basis)	LYSINE UTILIZED
	gm/100 gm ration	%
Lactalbumin	2.2	61 ± 5 [1]
Lactalbumin	2.9	60 ± 10
Beef round	2.3	92 ± 9
Beef round	3.0	83 ± 8
Beef round	2.0	84 ± 6
Beef round	3.0	90 ± 10
Beef rib	0.8	96 ± 24
Beef rib	1.7	70 ± 13
Beef rib (cooked)	1.1	68 ± 11
Beef rib (cooked)	2.3	71 ± 12

[1] Standard error.

TABLE 5

Per cent lysine utilized with amino acid and sesame meal basal rations

PRODUCT TESTED	AMINO ACID RATION [1]		SESAME MEAL RATION	
	Exp. I	Exp. II	Exp. I	Exp. II
Casein		71	81	
Wheat germ		57	72	
Beef round	87	86		88
Soybean oil meal		89	63	
Split peas		104	73	69
Pork (cooked)			72	74
Luncheon meat			78	60

[1] Results calculated from the 4th to 11th (or 5th to 12th) days on experiment (see text).

was not satisfactory. The reasons for these discrepancies are not readily apparent but may be attributed to variations associated with the use of a limited number of animals in each group.

These experiments have provided a background for evaluating the utilization of amino acids by the protein-depleted rat. They provide a sufficient range of response, permit the re-use of the same animals for a subsequent experiment and are of short duration. Certain limitations besides the cost of the amino acids are recognized. The percentage of the amino acid utilized from the test products is necessarily based on the standard response to graded levels of the limiting amino acid added in purified form. The amino acids used for protein synthesis of the depleted animal are provided in part as purified amino acids and in part as different proteins. These limitations should be kept in mind in the application of any technique for measuring the total nutrient potency (*cf.* Scheid et al., '52) or the utilization of nutrients biologically.

Other experiments were conducted to apply these techniques to estimation of the percentage of lysine utilized from certain purified proteins and foods by the protein-depleted rat. In addition, the lysine excretion in the feces was determined. The results of these tests are presented in the accompanying paper.

SUMMARY

Techniques were developed for determining the quantitative utilization of lysine from purified proteins and foods, using the protein-depleted rat as the test animal. Rations that contained purified amino acids or sesame meal plus diammonium citrate and certain amino acids were used as the basal rations deficient in lysine. The weight gain of the depleted rats fed these basal rations plus graded levels of lysine, or known levels of lysine in foods, was used as the basic criterion for estimating the lysine utilization from foods. The repeatability of the results in experiments replicated with time, with the use of different levels of the test product,

or with different basal rations, indicated that this approach could be used for studying the utilization of amino acids from foods. The limitations of the methods are also discussed.

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UTILIZATION OF AMINO ACIDS FROM FOODS BY THE RAT [1]

II. LYSINE

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Use of the protein-depleted adult male rat as a test animal for studying the quantitative utilization of lysine and the techniques necessary to carry out such experiments have been described in the previous paper (Schweigert and Guthneck, '53). Through application of the methods studied, it was possible to determine the per cent utilization of lysine by the rat from several test products. Among those tested were purified proteins, animal protein products, cereal foods and legumes. The results of these studies are reported in the present paper.

EXPERIMENTAL AND RESULTS

The fresh, cooked and processed meats employed were lyophilized, and the fat removed by ether extraction. The purified proteins, cereal and legume products were used as received. All test products were ground in a hammer mill and incorporated into the rations in a dried form to provide

[1] We are indebted to E. I. DuPont de Nemours and Company for supplying the L-lysine hydrochloride and DL-methionine, to the Dow Chemical Company for DL-tryptophan, to Merck and Company for the vitamin B_{12}, to the Lederle Laboratories Division, American Cyanamid Company, for the pteroylglutamic acid, to the Pacific Vegetable Oil Corporation for the sesame meal and to the Archer-Daniel Midland Company for the unheated soybean flakes used in this study.

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known amounts of lysine (150 to 350 mg/100 gm of ration) as determined microbiologically using the test organism *Leuconostoc mesenteroides* P-60. The techniques used in the animal experiments, preparation of the basal rations, and so forth, were the same as those outlined in the accompanying paper.

As a further test of the utilization of lysine from foods, based on the rates of gain for groups fed purified supplements, the amount of lysine excreted in the feces of certain groups was determined. A composite collection of the feces was made for each group once a day for a 4-day period in 150 ml of 3 N HCl. After the last collection, each composite was autoclaved for 16 hours at 15 lb. pressure, cooled, neutralized and diluted to the proper proportion for determination of lysine microbiologically using *Leuconostoc mesenteroides* P-60 as the test organism.

The excretion data for the groups fed graded levels of L-lysine and several of the test products with the sesame meal basal ration appear in table 1. Only one set of data was obtained with the amino acid basal ration, and not all the lysine-supplemented groups were included in the collection. The amount of lysine excreted by the groups receiving no lysine supplement was subtracted from the amount of lysine excreted by all other groups, receiving lysine either in the purified form or as present in a test product. The fecal samples for each group were collected from the 5th through the 9th days, during which period the rates of gain and food consumption data were also obtained. From this information the per cent lysine excreted was calculated, based on the ratio between the milligrams of lysine excreted, minus those for the control, and the milligrams of lysine ingested.

Certain limitations of this method of calculating the percentage of lysine excreted in the feces and attributable to ingestion of the test supplement are recognized. With the sesame meal ration a known, but varying, amount of lysine is contributed to the diet by the sesame meal. The effect of additional lysine on the "basal excretion" of lysine that

should be attributed to the sesame meal ingested cannot be ascertained. It will be noted that an increased excretion of lysine occurred when the L-lysine supplements as well as the test products were fed. The figures presented for the lysine excreted, therefore, are based on a combination of the lysine contributed by the sesame meal consumed, over and above

TABLE 1

Fecal excretion of lysine by rats fed lysine supplements and test products with the amino acid and sesame meal basal rations

SUPPLEMENT PER 100 GM RATION	AMOUNT	AMINO ACID RATION			SESAME MEAL RATION		
		Lysine excreted/rat	Lysine ingested/rat	Lysine excreted	Lysine excreted/rat	Lysine ingested/rat	Lysine excreted
	gm/100 gm ration	*mg*	*mg*	*%*	*mg*	*mg*	*%*
No supplement		14			64 66	89 111	
100 mg L-lysine					85 [1] 90	155 218	13.6 11.1
150 mg L-lysine					96 102	257 278	12.3 12.8
225 mg L-lysine					111 107	328 319 [2]	14.4 12.9
300 mg L-lysine		22 [2]	363	2.2	89 111	371 434	6.6 10.3
Rolled oats	15.1				94 110	236 274	12.7 15.9
Split peas	8.2	70	263	21.5	102 110	241 260	15.7 17.1
Soybean oil meal	4.5	43	242	12.2	93	216	13.4
Pork luncheon meat	2.3				82 88	257 254	7.0 8.7
Beef round	1.9	20	152	4.3	88	256	8.7
Beef rib (cooked)	1.7				79 96	244 273	6.1 11.0

[1] Supplement equivalent to 75 mg L-lysine per 100 gm ration.
[2] Supplement equivalent to 450 mg L-lysine per 100 gm ration.

that ingested by the control group, and on that contributed by the test supplement. Since the total lysine ingested when the test products were fed varied within narrow limits (216 to 274 mg per rat), the relative values presented are assumed to be valid. This conclusion is further attested to by the similarity in the values observed when the amino acid ration (devoid of added lysine) was fed.

The excretion data (tables 1 and 2) indicate a higher percentage of lysine excreted by animals fed the cereal-type foods (21.5, 15.7 and 17.1% for split peas and 12.7 and 15.9%

TABLE 2

Per cent lysine utilized for gain, the amount excreted in the feces and the total accounted for when various test products were fed

PRODUCT	PROTEIN IN DRIED SAMPLE	LYSINE IN DRIED SAMPLE [1]	UTILIZATION [2]	LYSINE EXCRETED	TOTAL ACCOUNTED FOR
	%	%	%	%	%
Casein	91.2	7.7	76 ± 8 (2)	7.6	84
Lactalbumin	73.8	9.3	60 ± 5 (1)		
Skim milk (dried)	33.1	3.1	83 ± 5 (2)	13.1	96
Whole eggs (dried)	47.8	3.7	98 ± 15 (1)	11.2	109
Wheat germ	34.6	2.1	65 ± 8 (2)	14.0	79
Rolled oats	16.8	0.86	77 ± 9 (2)	14.3	91
Split peas	22.9	1.9	81 ± 6 (3)	16.4	97
Soybean oil meal	51.5	3.2	76 ± 8 (2)	12.8	89
Soybean grits	51.9	3.5	80 ± 7 (2)	16.4	96
Soybean flakes [3]	48.9	3.7	49 ± 8 (1)	19.1	68
Pork and beef (canned)	66.3	7.2	71 ± 9 (2)	11.7	83
Pork luncheon meat	55.7	6.3	69 ± 7 (2)	7.8	77
Pork ham	89.3	8.8	82 ± 7 (2)	11.2	93
Pork ham (cooked)	92.2	8.9	73 ± 9 (2)	9.7	83
Beef round	88.1	7.8	87 ± 4 (3)	6.5	93
Beef rib	89.9	8.6	88 ± 9 (2)	14.9	103
Beef rib (cooked)	90.1	8.7	68 ± 6 (2)	8.5	76
Lamb leg	87.5	8.2	80 ± 5 (1)	11.6	92
Lamb leg (cooked)	89.5	9.1	96 ± 8 (1)	9.0	105

[1] Percentages of lysine in the dried sample represented at least 7 analyses. The protein analyses were made by the Service Laboratory, American Meat Institute Foundation.

[2] Unheated, solvent-extracted.

[3] Mean and standard error. The number of tests conducted with each product is indicated in parentheses.

for rolled oats) than by those fed meat products. Kuiken and Lyman ('48) also observed that small amounts of lysine ingested as part of roast beef were excreted in the feces by young, growing rats. A good correlation was obtained between the excretions for the same test product with the amino acid and sesame meal basal rations (i.e., rats fed soybean oil meal excreted 12.2 and 13.4%, respectively).

The total per cent of lysine that can be accounted for from the test products (derived from the average amount utilized for gain and the average amount excreted) is presented in table 2. The per cent utilization ranged from 49 to 98 for the different foods, and the total accounted for ranged from 68 to 109%. It is also of interest to note that there was greater utilization of lysine by the rat, as was found to be true of tryptophan for chick growth (Schweigert, '48), from casein and processed soybean meal than from unheated soybean flakes. Of the products tested, the unheated soybean flakes gave the lowest lysine utilization (49%) and highest excretion (19%). However, the amount of lysine utilized was much higher than that observed for tryptophan with the chick (20%).

The utilization of lysine from uncooked meats, dried eggs and skim milk ranged from 80 to 98%, while that from the purified proteins and cereal foods ranged from 60 to 82%. The cooked and processed meats showed a per cent utilization of from 68 to 96. A tendency for lower utilization was indicated from the cooked and processed cuts as compared to the uncooked meats, without a greater percentage of lysine being excreted. The lysine content of lamb leg appeared to be more readily utilized by the animal after cooking (96%) as compared to before cooking (80%). However, these results were not consistent enough to make it possible to detect small differences in utilization due to processing (see also accompanying paper).

SUMMARY

The per cent lysine utilized by the protein-depleted rat from purified proteins, animal protein products, cereal and

legume foods was determined. On an over-all basis, the results indicate a greater utilization of lysine from dried eggs and skim milk (91%) and from fresh meats (84%) than from cereal and legume foods (71%) and cooked or processed meats (70%). The fecal excretion of lysine was determined, and the results showed that a greater amount of lysine was excreted in the feces of animals fed the cereal foods and unheated soybean flakes than of those fed other test products.

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THE RELATIVE CARIOGENICITY OF SUCROSE WHEN INGESTED IN THE SOLID FORM AND IN SOLUTION BY THE ALBINO RAT [1]

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Various studies have indicated that table sugar (sucrose) was a potent cariogenic factor in the purified diets that have been used to induce caries in the Norway rat (Sognnaes, '48; Haldi and Wynn, '52). The present study was undertaken to determine whether sucrose in the solid form is more or less cariogenic than sucrose in solution. This was possible through a slight modification of the technique employed by Kite, Shaw and Sognnaes ('50), who fed rats a complete diet by stomach tube from the time of weaning through an experimental period of 20 weeks. The present modification, where part of the diet is tube-fed and the remainder ingested orally, affords a suitable means for assessing the local etiological action of individual food items in the production of dental caries. By following that technique in this investigation, we were able to provide our experimental animals with adequate nutriment, and at the same time allow no food whatsoever to come into direct oral contact with the teeth of the rats in one group, and only sucrose in the solid form or in solution to come in contact with the teeth of the rats in other groups.

[1] The studies described in this paper have been supported to a considerable extent by grants-in-aid to the two cooperating laboratories by the Sugar Research Foundation, Inc.

EXPERIMENTAL

Twenty sets of albino rats, each set consisting of three litter mates, were selected at weaning from the colony of the Wistar strain in the Physiology Laboratories of the Emory University School of Dentistry. Weanling animals from this colony, when placed on a high sugar ration, have proved sufficiently caries-susceptible to develop some carious lesions, although their parents had been fed a stock diet. In another investigation it was observed that the purified diet used in the Harvard studies (Sognnaes, '48) was more cariogenic than the Emory diet (unpublished data). For this reason, and because of the greater ease of feeding the Harvard purified diet by stomach tube, it was chosen for these experiments. The composition of this sugar-containing diet has been described previously (Shaw, '47).

Immediately after weaning, the 20 trios of rats were distributed into three groups, with each trio having a representative in each of the three groups. For the remainder of the experiment, a period of 90 days, the control animals (group I) were fed the complete sugar-containing diet by stomach tube. All rats in groups II and III were fed by stomach tube the same purified diet except that the sucrose had been completely omitted. In addition, the rats in group II were given weighed amounts of granulated sugar in a food cup. The rats in group III received measured amounts of a 40% sugar solution in a test tube with a delivery tube attached to the side of the cage. The daily ration of sucrose, both in the granulated form and in solution, was adjusted so as to make the sugar intake of the experimental animals in groups II and III the same as that of the control animals (group I) which were fed the entire food mixture by stomach tube. Likewise the intake of the non-sugar portion of the diet was maintained at equal levels for the experimental and control rats.

The procedure described by Levin ('42) was used for the stomach tube feeding, with a few modifications. This method permits the entire operation to be carried out by one person.

After one becomes adept with the procedure, an animal can be removed from the cage, fed by stomach tube, and returned to the cage in less than a minute.

The animal is held with its back against the palm of the left hand. The thumb and index finger of the operator are clasped snugly but not tightly around the animal's shoulder. The animal's right arm is held against its body by the operator's left thumb and its left arm between the thumb and second finger. A loop at the end of a string about 20 inches long is caught over the upper incisors. The string is passed over the back of the left hand and between the 4th and 5th fingers and fastened by being wound several times around the latter. A loop at the other end of the string is caught over the lower incisors, passed down over the back of the thumb and fastened by being wound around the little finger of the left hand. The mouth can then be opened by gently pulling apart the thumb and index finger.

Of the several kinds of tubing we tried, we found most satisfactory a transparent, transflex plastic tubing, No. 16, with an inside diameter of 0.053 inch.[2] This tubing fits snugly over a No. 15 syringe needle which has been cut off to a length of about one inch.

While the tube feeding process was being conducted, the food mixture suspended in water was kept close at hand in a small beaker. It was held in suspension by constant stirring with an electric stirrer and drawn into the syringe as needed.

Water was added to the food mixtures in such amounts that 1 ml of the suspension of the sugar-containing diet provided 1 gm of the food mixture and 1 ml of the sugar-free diet suspension $\frac{1}{3}$ gm. In each milliliter of sugar-containing diet there was $\frac{2}{3}$ gm sugar. In order to equalize the food intake of the experimental and control animals, the former were given $\frac{2}{3}$ gm sugar, either in the solid or liquid form, for each milliliter of the suspended food mixture given by stomach tube. All the animals were fed by stomach tube three times

[2] Irvington Varnish and Insulation Company, Irvington, New Jersey.

a day. The first three or 4 days after weaning, they were given 1 ml of the food suspension at each tubing; then for one week 2 ml per feeding; the following week 4 ml; and from then on, 5 ml for the remainder of the experiment.

After the animals had been on the experiment for 30 days, they were desalivated in order to accelerate the carious process. Then the usual feeding procedure was continued for an additional 60 days. For the purpose of surgical desalivation, the rats were anesthetized by intraperitoneal injection of 4 mg sodium nembutal per 100 gm of body weight. An incision was made in the neck and the underlying structures exposed. The parotid duct and external jugular vein on each side were tied with a silk ligature and resected. The ducts of the submaxillary and major sublingual glands on each side and the accompanying blood vessels were also ligated and cut. The submaxillary glands, the major sublinguals and most of the parotid on each side were dissected out by teasing with a probe. A small portion of the parotid located down near the shoulder was not removed. The exorbital lachrymal gland on each side was dissected free and excised. There was very little loss of blood, and the animals appeared to suffer no ill effects from the operation.

At the end of the experiment, the animals were decapitated and the upper and lower jawbones removed. One quadrant of the upper jaw and the corresponding quadrant of the lower jaw were fixed in 4% basic lead acetate for 24 hours. They were then rinsed in distilled water and placed in 10% neutral formalin. The other two quadrants, upper and lower, were fixed immediately in 10% neutral formalin. The teeth were later examined under a dissecting microscope at a magnification of 30 diameters for the number and extent of carious lesions. Since the jaws were desired for histological studies, the caries examination was made without any grinding away of the occlusal surfaces. This has been found to be permissible in the white rat by reason of the wide and relatively shallow sulci of the rat molar as compared to those in the cotton rat. The criteria for judging and evaluating the size

of the carious lesions were those described by Shaw, Schweigert, McIntire, Elvehjem and Phillips ('44).

RESULTS

Kite, Shaw and Sognnaes ('50) have pointed out the difficulties associated with feeding young rats by stomach tube, and have described the various factors probably responsible for the fatalities which may occur. Of the 60 animals with which we started the experiment, 13 were lost within the first few days and 13 more shortly after desalivation.

The animals that survived had normal growth and manifested no evidence of malnutrition. At the conclusion of the experiment — 90 days after weaning — the average weight of the three groups of females was 196, 182 and 200 gm, respectively, or an increase over their original weight of 301, 304 and 334%; the average weight of the males was 231, 232 and 252 gm or an increase of 372, 382 and 394%, respectively. These gains are of the same order of magnitude as those we have observed in other experiments in which the animals were allowed to eat the Emory high sucrose diet ad libitum. In scrutinizing the difference in average weights of the control and the two experimental groups despite the equalization of food intake, it should be borne in mind that some of the animals were lost and these weights consequently are not average weights of litter mates.

Gross dental findings

The average number of carious molars, the average number of carious lesions, and the average extent of carious lesions for the three groups in this experiment are presented in table 1. The smaller number of animals in group I at the conclusion of the experiment, as shown in the table, was due to a higher mortality in this group. This is accounted for by the greater difficulties that were encountered in feeding the entire ration by stomach tube. As we have noted in our laboratories, in keeping with the observations of others

(Sognnaes, '49), that there is no sex difference in caries susceptibility in the Norway rat, only the composite averages from the data on males and females are presented in the table.

Each of the 8 rats in group I, which received the complete diet by stomach tube, was completely free of any carious lesions which could be detected by detailed examination at a magnification of 30 diameters. Eleven of the 13 rats in group II, which received granular sucrose by mouth and the rest of the diet by stomach tube, had one or more definitely

TABLE 1

Dental caries incidence in albino rats when sucrose was fed by various methods

GROUP	METHOD OF FEEDING	NO. OF RATS	AVERAGE WEIGHT INCREASE		NO. OF CARIES-FREE RATS	AVE. NO. OF CARIOUS MOLARS	AVE. NO. OF CARIOUS LESIONS	AVE. CARIES SCORE
			Male	Female				
			gm	*gm*				
I	Entire ration by stomach tube	8	231	196	8	0	0	0
II	Granular sucrose ingested orally (remainder of ration by stomach tube)	13	232	182	2	3.8	4.9	7.5+
III	Sucrose in solution ingested orally (remainder of ration by stomach tube)	13	252	208	7	0.8	0.8	1.2+

Critical ratios[1]

GROUPS	AVE. NO. OF CARIOUS MOLARS	AVE. NO. OF CARIOUS LESIONS	AVE. CARIES SCORE
I vs. II	5.4	5.4	4.2
I vs. III	2.7	2.7	2.4
II vs. III	3.9	4.7	3.4

[1] *Critical ratio:* The critical ratio is the ratio of the difference between two means to the standard error of the difference between the means. Whenever the critical ratio is less than 2.0, the difference between the means is considered to be statistically insignificant; whenever the critical ratio is between 2.0 and 2.9, the difference is of borderline significance; when the critical ratio is 3.0 or higher, the difference is highly significant.

carious lesions, some of which were well advanced in their stage of development. Only 6 of the 13 rats in group III, which were allowed the same amount of sucrose per day in solution by mouth but the rest of the diet by stomach tube, had small and infrequent carious lesions. Grossly, the carious lesions in the molars of the rats in groups II and III were typical of those lesions previously observed in rats of the same strain which were maintained on the same diet ingested orally. The difference in size and rate of progress between the lesions of the rats in groups II and III was clearly evident in the examination of the intact teeth. The carious lesions in the rats in group II were uniformly larger, softer, lighter in color, and thereby presumably more rapidly progressing, than the lesions found in the rats of group III.

The difference in caries incidence between the animals in groups I and II was found to be highly significant by the critical ratio test whether applied to the number of carious molars, the number of carious lesions, or the caries score. The difference in caries incidence between the rats in groups II and III was also highly significant for the above three criteria. The difference in caries incidence between the rats in groups I and III was borderline in statistical significance. The latter fact, coupled with the observation that just over half of the rats in group III were entirely caries free, leads to the conclusion that in these experiments sugar in solution was much less cariogenic than dry, granular sugar.

Histological findings

Ground sections and decalcified sections of the jaws and teeth of representative animals were prepared for microscopic examination. The purpose of this examination was to determine the presence of impactions and microorganisms within the fissures of the molar teeth, the location of debris in relation to the enamel surface, the type of carious lesions, and the general appearance of the enamel, dentin, pulp and supporting structures of the teeth.

The group which received the complete diet by stomach tube, and nothing but water orally, presented histological features similar to those observed in the previous tube feeding experiment of Kite, Shaw and Sognnaes ('50). The fissures contained moderate amounts of debris, which presumably had collected there during the pre-experimental period and had been retained throughout the entire experiment. The deeper portions of the fissures of the molar teeth were relatively free from microorganisms. However, the persisting cuticular lining of the enamel which was apparent in the tube feeding experiment referred to above was not as distinct, and some microorganisms had established themselves in a few areas below impacted detritus in the sulci of the molars. No explanation for these minor differences is known, for to the best of our knowledge the procedures followed with the present group and the tube-fed group in the previous experiment were identical. Notwithstanding these observations, the enamel and dentin proved to be surprisingly intact at the microscopic level.

In animals in group II which were allowed to eat granular sugar by mouth, the ground and the decalcified sections showed definite cavities in the depth of the fissures, accompanied by surrounding pigmentation and staining of the enamel and dentin. Furthermore, the fissures were filled with microorganisms. No cuticular lining remained on the enamel surface, and invasion of the microorganisms could be observed between the enamel prisms and into the dentin to various distances. Even the interproximal surfaces of the enamel as studied in ground sections were surrounded by a multitude of microorganisms; the enamel surface adjoining these areas proved to have an increased permeability to stains, indicating some loss of the inorganic substance and resulting in an unmasking of the organic matter.

Studies of the teeth of the rats in group III, when the sucrose allowance was consumed in a 40% aqueous solution, indicated that the lesions had all the characteristics of those in the teeth of the rats in group II, with a single exception.

The degree of penetration into the tooth substance was appreciably less, which was highly suggestive of a reduced caries attack rate in this group of rats.

In all three groups of rats, the periodontal structures were judged to be normal. The pulp was also normal in every case except in those instances in group II where the carious lesions had penetrated sufficiently to produce a typical pulpal involvement.

DISCUSSION

In the experiments of Kite, Shaw and Sognnaęs ('50) it was found that in the strain of Norway rats raised in the Harvard Biological Laboratories, the Harvard high sucrose cariogenic, synthetic ration had to come into direct oral contact with the teeth in order for it to produce carious lesions even in desalivated animals. These observations are confirmed by the present study on the albino rat of the Wistar strain, in which it was found that no carious lesions developed when this highly cariogenic diet was fed by stomach tube. The cariogenicity of the diet when fed after weaning must therefore be attributed to a local or environmental effect on the teeth and not to a systemic action. It should be noted, however, that when the diet is fed during the period of early tooth development, it may exert an influence on later susceptibility to caries (cf. Sognnaes, '48).

Sugar has long been suspected of playing an important role in the etiology of dental caries. Sognnaes ('48) has shown that the sugar component which constitutes 67% of the synthetic ration used in these experiments is at least an important factor contributing to its cariogenicity, for the replacement of sucrose by dextrin or whole wheat flour resulted in a much lower incidence of carious lesions. While we recognize that at the present stage of dental investigation it is not judicious to apply the results of experiments on rats' teeth directly to human teeth, nevertheless it is highly desirable to study the way in which circumstances, similar to those that obtain in human dietary practices, may modify

the cariogenic action of sugar on rats' teeth. Sugar in the human dietary is consumed both in the solid form and in solution, as in milk, coffee, tea and other beverages.

Schweigert, Shaw, Zepplin and Elvehjem ('46) and Anderson, Smith, Elvehjem and Phillips ('48) observed that fluidity of the ration was an important factor in dental decay in the cotton rat. The rats which received the solid rations in these experiments had a higher dental caries attack rate than comparable groups which received the same diets in fluid form. Our experiments, which go a step further in studying a single component of the ration with respect to its physical form, lead to similar conclusions. Anderson and her associates were seeking an explanation for the fact that liquid whole milk, which contains the sugar, lactose, did not produce carious lesions in the cotton rat. Whole milk was found to offer almost complete protection against caries even in the presence of an added 5 or 10% of fermentable sugar. The latter observations also obtain with respect to the albino rat (Shaw, '50).

Fluidity is obviously an important factor, as is evidenced by the fact that the ingestion of a dry whole milk mixture resulted in a number of carious lesions, whereas caries became almost negligible when the mixture was reconstituted with water to produce a liquid ration equivalent to whole milk. However, the authors of these studies recognized that there were other factors besides fluidity which might have contributed to their results; namely, the presence of fat in liquid milk and a possible difference in the physical state of fat in dry whole milk and in liquid milk. They call attention to the postulate of other investigators that free fat as oil in the diet may reduce dental caries by forming a mechanical coating over the tooth or food particle, thus protecting against bacterial action.

In our experiments no other food but sugar came into contact with the external surfaces of the teeth, and the only variable was the physical state of the sugar fed orally. It should be noted that conditions conducive to dental decay were most severe because the animals had been desalivated.

Yet in spite of the severity of these conditions, the number of carious lesions and the caries score, from the absolute point of view, were very low when sugar was ingested in solution. On the other hand, these same values, which are indicative of the incidence and extent of caries, were relatively high when sugar was eaten in the solid form. It therefore becomes obvious that the cariogenicity of sucrose in these experiments was greatly reduced by dissolving the sugar in water before ingestion.

SUMMARY AND CONCLUSIONS

Albino rats of the Wistar strain developed no carious lesions when they were fed entirely by stomach tube a high sucrose diet which had previously been shown to be cariogenic when ingested orally.

When all the components of this food mixture except sugar were fed by stomach tube and granulated sugar was ingested orally, the animals having been desalivated for the greater part of the experiment, there developed an appreciable number of carious areas and an appreciable caries score.

When, under the same experimental conditions, sugar was ingested in a 40% aqueous solution, the number of carious areas and the caries score were markedly less than when the same amount of sugar was eaten in the solid form. More than half of the rats were entirely caries-free.

Sugar in solution was therefore found in these experiments to be much less cariogenic than granulated sugar.

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POSSIBLE MECHANISM INVOLVED IN THE GROWTH-PROMOTING RESPONSES OBTAINED FROM ANTIBIOTICS

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It has been reported that aureomycin, streptomycin, penicillin and sulfasuxidine promoted the growth of chicks adequately supplied with vitamin B_{12} (Stokstad and Jukes, '50; Whitehill et al., '50; Briggs, '50; Groschke and Evans, '50). Reed and Couch ('50) failed to obtain an increase in the growth of chicks kept on sand litter by adding an animal protein factor (APF) concentrate, which contained only vitamin B_{12}, to an all-vegetable-protein diet, but obtained a very significant increase in chick growth by adding an APF concentrate obtained from *Streptomyces aureofaciens* fermentation. Elam, Gee and Couch ('51a, b) reported that the fecal microflora of the growing chick was influenced by the feeding or injection, or both, of penicillin and bacitracin. These workers reported further that penicillin and aureomycin increased egg production and hatchability. Matterson and Singsen ('51), working with chicks, and Carpenter ('51) with swine, observed that aureomycin, terramycin, streptomycin, penicillin and other antibiotics stimulated the growth rates of these experimental animals.

Elam, Gee and Couch ('51b) reported that antibiotics may possibly stimulate growth by some other mechanism in addition to action on the aerobic microflora. Larson and Carpen-

ter ('52) reported that aureomycin, administered either orally or intraperitoneally, is excreted in the feces of pigs. These workers postulated that injected aureomycin enters the intestinal tract of the pig through the bile. Rosenberg et al. ('52) reported that terramycin did not have any effect on the aerobic microflora with the exception of enterococci counts, which were significantly greater when high levels of terramycin were fed.

Kiser et al. ('52) reported that a number of clostridia were destroyed, in *in vitro* studies, by the addition of aureomycin, penicillin or terramycin. The growth-promoting effect of penicillin and terramycin has been explained as an inhibition of *Clostridium perfringens* in the intestinal tract of turkeys by Sieburth et al. ('51).

Several possible explanations have been offered for the growth-promoting properties of antibiotics. One of these explanations is the inhibition of toxin-producing bacteria. The experiments described in this paper were conducted to determine the effects of antibiotics on the total number of clostridia in fecal material from birds fed such drugs.

EXPERIMENTAL

Data from two separate experiments are reported in the present study. New Hampshire chicks, used in these studies, were obtained from hens that had been fed the diet described earlier by Elam et al. ('51b). The chicks were maintained in batteries with raised screen floors. Feed and water were supplied ad libitum and the chicks were weighed at weekly intervals during an experimental period of 10 weeks.

The basal diet used in this study was the same as that reported earlier by Couch and Olcese ('50), except that riboflavin, calcium pantothenate, niacin and choline were fed at levels of 2, 5, 12.5, and 200 mg per pound of feed, respectively, and vitamin B_{12} was mixed into the diet at a level of 30 μg per kilogram of diet.

Level of supplements added

Antibiotics were fed at a level of 10 gm per ton of feed for aureomycin, bacitracin and terramycin, whereas penicillin was administered at a level of 4 gm per ton of feed. In the second experiment, when the antibiotics were fed in combination, the total amount of antibiotic fed never exceeded 10 gm per ton of feed. Penicillin and inactivated penicillin (autoclaved for 15 minutes at 15 pounds) were injected at a level of 1.2 mg per bird per week. Sodium potassium benzylpenilloate, sodium benzylpenicilloate, and beta-diethylaminoethanol were administered parenterally at a level of 1.5, 1.2, and 0.43 mg, respectively, per bird per week. Arsanilic acid and sodium arsanilate were fed at a level of 90 gm per ton of feed and added at a level of 45 gm per ton when fed in combination with an antibiotic.

Number of birds used

For the first experiment 450 chicks were randomized into 18 groups of 25 chicks each and in the second experiment 500 chicks were randomized into 20 groups of 25 chicks each.

Bacteriological techniques used

Fecal samples were collected once a week and were prepared as described by Elam, Gee and Couch ('51a). A sulfite-glucose-iron agar, previously described by Wilson and Blair ('24) and Skinner and Baskin ('32), was used for determination of the anaerobes (primarily, the genus *Clostridium*). The plates were incubated anaerobically at 37°C. for 48 hours in Brewer anaerobe jars. After this time all colonies were counted. The colonies were then transferred to a thioglycollate medium [1] to obtain stock cultures. Colonies were then run through a series of standard bacteriological tests to determine whether they were strict anaerobes or merely aerobes which grew anaerobically.

[1] Baltimore Biological Laboratories.

In vitro studies were run on the strict anaerobes isolated from the control group to determine the amount of penicillin, aureomycin, bacitracin and inactivated penicillin required to inhibit their growth.

Statistical methods used

An analysis of variance for counts and weights was made, and the F value (ratio of appropriate mean square values from an anlysis of variance table) was determined. Least significant differences (LSD) were calculated where the F value was significant at the 1 or 5% level of probability.

RESULTS AND DISCUSSION

First experiment

The addition of penicillin to the basal diet (orally or parenterally) produced an increase in the growth of the chicks (table 1). The administration of inactivated penicillin parenterally produced a similar increase but failed to do so when fed orally. Growth was likewise increased by the feeding of aureomycin and bacitracin and by the feeding of combinations of penicillin and aureomycin, and bacitracin and penicillin. The increase in growth observed by the addition of 3% dried whey to the basal diet (table 1) was within the range of that produced by the addition of antibiotics without dried whey (table 1). A further increase in growth was apparent when penicillin was fed or injected, or when penicillin was fed with aureomycin or with bacitracin. Such an observation leads to the conclusion that a synergistic relationship exists between penicillin and dried whey. Reed, Atkinson and Couch ('51) presented data which showed a synergistic relation between penicillin and dried whey in tests with chicks maintained under practical conditions on litter and in contact with fecal material.

The fact that aureomycin, bacitracin and inactivated penicillin (orally or parenterally) failed to produce an increase in the growth of the birds in the presence of dried whey is not

an unusual finding. Unpublished data from this laboratory have been obtained from a number of experiments where antibiotics did not always produce an increase in growth when added to the diet of chicks kept in batteries with raised screen floors.

Administration of penicillin (orally or parenterally), inactivated penicillin (parenterally), and combinations of peni-

TABLE 1

First experiment

The effect of feeding antibiotics with and without 3% dried whey (50% lactose) upon the weights and the anaerobic microflora *(Clostridium)* of New Hampshire chicks[1]

GROUP	WITH WHEY		WITHOUT WHEY	
	Average weight	Total number of *Clostridium* isolates	Average weight	Total number of *Clostridium* isolates
	gm	*per gm feces*	*gm*	*per gm feces*
Control	1252	12,450	1153	45,580
Penicillin (oral)	1392	138	1295	275
Penicillin (inj.)	1338	850	1242	300
Inactivated penicillin (oral)	1256	1,250	1179	1,100
Inactivated penicillin (inj.)	1242	563	1257	100
Aureomycin	1270	100	1278	125
Bacitracin	1284	2,812	1286	100
Penicillin + aureomycin	1371	100	1335	100
Bacitracin + penicillin	1391	100	1263	150

[1] Amount of antibiotic administered: penicillin 2 mg/lb., aureomycin 5 mg/lb., bacitracin 5 mg/lb., inactivated penicillin 2 mg/lb. The injected antibiotics were administered at a level of 1.2 mg per bird per week.

cillin and aureomycin, and bacitracin and penicillin produced a very significant decrease in the total number of fecal clostridia. The decrease in clostridia produced by the feeding of inactivated penicillin was also significant but was of a lesser magnitude than that which occurred through the injection of inactivated penicillin. It was pointed out above that the oral feeding of inactivated penicillin failed to increase the growth of the birds. There is little question but

that the inactivated penicillin given orally or by injection decreased the number of clostridia in fecal material. Apparently all of the antibiotic activity of the penicillin was not destroyed, although detection was not possible by standard penicillin assays (cup plate and disc technic methods). It should be pointed out that 1 μg of procaine penicillin, aureomycin, or bacitracin per milliliter of inoculated medium inhibited the growth of isolated clostridia *in vitro*, whereas the addition of 1,000 μg per milliliter of inactivated penicillin failed to produce such an inhibition. It is possible that the inactivated penicillin might have been reactivated within the body of the bird and excreted into the digestive tract, after which an effect on the clostridia was produced. It is possible also that a fragment of the penicillin molecule might have inhibited the clostridia in the gastrointestinal tract. Further data on the derivatives of penicillin are given in the second experiment described below.

Attention is directed to the fact that the addition of dried whey to the basal diet (table 1) produced a decrease in the number of clostridia per gram of fecal material, although the decrease was not statistically significant. A definite decrease in the number of clostridia was produced by the feeding of antibiotics in the presence of 3% dried whey. The data of this test are considered to be of particular significance, since a close relationship has been shown to exist between the administration of an antibiotic, the reduction of the number of clostridia per gram of feces, and an accelerated growth rate. Such an observation is in agreement with the earlier report of Sieburth et al. ('51), where the inhibition of *Clostridium perfringens* was given as an explanation for the growth-promoting effect of penicillin and terramycin. Attention is also directed to the paper of Kiser et al. ('52), in which it was reported that a number of clostridia were destroyed by the addition of aureomycin, penicillin or terramycin to the medium.

Second experiment

The growth obtained in the second experiment was not equal to that obtained in the first experiment (tables 1 and 2). It should be emphasized that the second test was carried out during the months of May, June and July. The weather was hot and the laboratory was not air-conditioned. The hot weather could have been responsible for the poorer growth obtained in the second experiment, in all groups, as compared to results of the first test.

The growth of the birds was increased by adding penicillin, aureomycin, bacitracin, terramycin, arsanilic acid or sodium arsanilate to the diet. The feeding of arsanilic acid with penicillin, or sodium arsanilate with this antibiotic, failed to produce an increase in growth over that obtained by feeding either of the arsenicals or penicillin alone. It is possible that the combination of the antibiotics and the arsenicals might have produced an increase in growth if the weather had been cooler and the growth which was observed in the first test obtained. Attention is directed to the fact that inactivated penicillin again produced an increase in growth when administered parenterally. Injection of beta-diethylaminoethanol produced 100% mortality within 24 hours after administration. The injection of this compound produced an immediate intoxication, which was followed by a swelling in the vicinity of the injected area. This area later turned dark and was characterized by a profuse hemorrhage. Death resulted very shortly thereafter. Sodium potassium benzylpenilloate failed to have any effect upon growth, although it did not produce toxic symptoms or mortality. Sodium benzylpenicilloate when administered parenterally produced toxic symptoms which consisted of mild intoxication and swelling and discoloration in the vicinity of the injected area. The injection of this compound resulted in 88% mortality during the course of this study.

The administration of combinations of antibiotics (table 2) failed to produce an increase in growth over and above that

TABLE 2

Second experiment

The effect of antibiotics, combinations of antibiotics, derivatives of penicillin, arsenicals, and combinations of arsenicals and penicillin upon the weights and anaerobic microflora *(Clostridium)* of New Hampshire chicks at 10 weeks of age

Group	Average weight	Total number of *Clostridium* isolates
	gm	*per gm feces*
Control	907	11,930
Penicillin (2 mg/lb.)	1056	233
Aureomycin (5 mg/lb.)	1064	392
Bacitracin (5 mg/lb.)	1011	423
Terramycin (5 mg/lb.)	1143	83
Arsanilic acid (45 mg/lb.)	1097	72
Sodium arsanilate (45 mg/lb.)	1029	1,402
Arsanilic acid (22.5 mg/lb.) + penicillin (1 mg/lb.)	1064	146
Sodium arsanilate (22.5 mg/lb.) + penicillin (1 mg/lb.)	1044	366
Inactivated penicillin (1.2 mg)[1]	1007	164
Sodium potassium benzylpenilloate (1.5 mg)[1]	902	5,750
Sodium benzylpenicilloate (1.2 mg)[1]	(88% mortality)	7,004
Beta-diethylaminoethanol (0.4 mg)[1]	(100% mortality)	
Penicillin (1 mg/lb.) + aureomycin (2.5 mg/lb.)	1032	360
Penicillin (1 mg/lb.) + bacitracin (2.5 mg/lb.)	1026	102
Penicillin (1 mg/lb.) + terramycin (2.5 mg/lb.)	1081	153
Aureomycin (2.5 mg/lb.) + bacitracin (2.5 mg/lb.)	1051	277
Aureomycin (2.5 mg/lb.) + terramycin (2.5 mg/lb.)	1054	93
Bacitracin (2.5 mg/lb.) + terramycin (2.5 mg/lb.)	1066	288
Penicillin (0.67 mg/lb.) + aureomycin (1.67 mg/lb.) + bacitracin (1.67 mg/lb.)	1077	391
Penicillin (0.67 mg/lb.) + aureomycin (1.67 mg/lb.) + terramycin (1.67 mg/lb.)	1071	21
Penicillin (0.67 mg/lb.) + bacitracin (1.67 mg/lb.) + terramycin (1.67 mg/lb.)	1114	138
Aureomycin (1.67 mg/lb.) + bacitracin (1.67 mg/lb.) + terramycin (1.67 mg/lb.)	1109	84
Penicillin (0.5 mg/lb.) + bacitracin (1.25 mg/lb.) + aureomycin (1.25 mg/lb.) + terramycin (1.25 mg/lb.)	1084	55

[1] Injected intramuscularly into each bird weekly.

obtained by the feeding of the antibiotics singly. The feeding of combinations of antibiotics, however, did increase the growth of the birds over that observed in the chicks fed the basal diet. Attention is again called to the fact that maximum growth was not obtained due to the hot weather during the months of May, June and July.

The number of clostridia per gram of feces was decreased in all instances in which antibiotics were fed singly or in combination. The feeding of the arsenicals, arsanilic acid and sodium arsanilate, likewise produced a significant decrease in the total number of clostridia per gram of feces. Attention is again directed to the fact that the parenteral administration of inactivated penicillin decreased the number of clostridia in fecal material. The injection of the penicillin derivatives produced a decrease in the number of clostridia, although it was not statistically significant.

The data of the second experiment confirmed the observations made in the first experiment to the effect that there is a close relation between an antibiotic, arsenicals, combinations of antibiotics, and a combination of an antibiotic and arsenicals and reduction in the number of clostridia per gram of fecal material.

The fact that antibiotics have been shown to increase growth in these studies is in agreement with earlier reports (Stokstad and Jukes, '50; Whitehill et al., '50; Briggs, '50; Groschke and Evans, '50).

The data of this paper are also in agreement with material which was presented at the Notre Dame Colloquium, June 4, 1952, Notre Dame, Indiana, to the effect that antibiotics exert growth-stimulating activity through an effect on the intestinal microflora. The workers concerned (Reyniers, '52; Luckey, '52; Gordon, '52) presented evidence showing clearly that antibiotics did not stimulate the growth of birds maintained under germ-free conditions, but that antibiotics did increase the growth of chicks maintained under ordinary laboratory conditions. They stated that the increase in growth which results from the feeding of anti-

biotics under laboratory conditions (not germ-free) must be associated with the presence of bacteria in the gastrointestinal tract. The data of this paper show that there is a decrease in the anaerobe content of fecal material from chicks fed antibiotics when compared to that of birds not fed these drugs.

SUMMARY

Data presented in this report show that the administration of an antibiotic produced a significant increase in growth and a significant decrease in the total number of clostridia per gram of feces.

Earlier reports are confirmed to the effect that penicillin, aureomycin, bacitracin, and terramycin stimulated the growth of chicks.

Arsanilic acid and sodium arsanilate have been shown to increase growth and decrease the total number of clostridia per gram of feces when fed to chicks.

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STUDIES ON OBESITY

I. NUTRITIONAL OBESITY IN MICE [1]

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THREE FIGURES

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In a previous paper (Fenton and Carr, '51b) it was reported that relatively young mice of certain strains occasionally became obese through the feeding of complete, highly purified diets. With the general recognition of the role of obesity in the etiology of a variety of human disorders, it seemed of interest to pursue this matter further. The ability to induce or prevent obesity at will by nutritional means alone would seem to furnish an excellent tool for the evaluation of the exact role of obesity in disease. It should be stressed that mice of many inbred strains become obese late in life without special nutritional manipulation. The obesity to be reported in this paper, however, has its onset between the third and 6th months of age. Furthermore, the strains used in this study are not usually thought of as becoming obese during these early months of life. Fenton and Carr ('51b) reported on the growth rates and efficiency of food utilization of 4 strains of weanling mice during 30 days on diets differing in fat content (5, 15, 25 and 47.5%). Mice of the

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Vitamins of the B complex were obtained through the courtesy of Hoffmann-LaRoche, Inc. Folic acid was furnished by the Lederle Laboratories Division, American Cyanamid Co.

C_3H and A strains showed weight gains roughly proportional to the fat content of the diet. On the other hand, C_{57} and I strain mice grew better on a 15% than on a 5% fat diet. Further increasing the fat content of the diet did not yield greater weight gains.

The present paper deals with the weight gain and fat deposition of this same group of mice observed until they were approximately 6 months old. In addition, data are presented on a much larger group of mice maintained on other

TABLE 1

Composition of diets[1]

INGREDIENTS	DIET									
	223	224	225	226	227[2]	241	242	245	257	273
	gm	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*	*gm*
Casein	30	90	45	30	30	10	5	30	32	30
Lactalbumin	..	..	..	..	..	..	85	..	5	..
Dextrin	60	..	..	..	50	..	..	40	..	15
Sucrose	..	..	..	17.5	..	80	..	..	..	..
Lactose	..	..	..	..	..	..	..	..	9	..
Corn oil	5	5	7.5	5	5	5	5	5	5	5
Crisco	..	..	40	42.5	10	..	..	20	44	45
Salts A[3]	5	5	7.5	5	5	5	5	5	5	5

[1] The vitamin content of these diets and the composition of salts A are those described by Fenton and Carr ('51a).

[2] Diet 254 was prepared by adding 10 gm of Ruffex to 90 gm of diet 227.

[3] See footnote 1.

diets. For the greater part, the paper deals with the effect on the development of nutritional obesity of changing the proportions of dietary protein, fat and carbohydrate. In addition, the chemical determinations of carcass fat seem to provide a useful criterion for evaluating the appearance and degree of obesity in the experimental animals.

METHODS

Male mice of 4 strains (C_{57}, I, C_3H and A) were weaned when 21 days old and placed on one of the experimental diets (table 1). More than 30 diets and over 600 animals

have been used in this work, but the results with only a few of these diets are reported here. Throughout the experiment food and water were supplied ad libitum. The animals were housed in individual screen-bottom cages in a constant temperature room. The mice were weighed every third day until they were 51 days old. Thereafter they were weighed once each week. At intervals during this period some mice were sacrificed for carcass analysis. Fat was determined by the method of Sinclair as described by Feyder ('35). Nitrogen was determined by the micro Kjeldahl method.

RESULTS

In figure 1 are shown the average weights of our 4 strains of mice fed a variety of diets differing principally in their content of protein, fat and carbohydrate. In the experiments reported here the body weight averages of C_{57} and I strain mice never exceeded 35 gm at 6 months of age. In fact, only a very few groups attained weights greater than 30 gm. On the other hand, mice of the C_3H strain attained final body weights greater than 35 gm in all but one case. Mice of the A strain became almost as large as those of the C_3H strain. Many of the C_3H and A strain mice appeared obese. With these animals the feeding of the high fat diet, no. 226, led to the greatest final body weight, while the 5% fat diet, no. 223, produced considerably poorer weight gains. Diets containing more than 15% fat appeared to inhibit the rate of weight gain of the I strain mice. With all 4 strains the poorest weight gains were obtained with diets containing 90% protein, diets 224 and 242. Feeding a 10% protein diet, no. 241, resulted in the following average body weights at 6 months of age: C_{57} — 24.1 gm; C_3H — 41.6 gm; A — 33.6 gm; and I strain — 24.0 gm. Since the high fat diets, such as diet 226, were consumed in smaller amounts than diets of lower fat content, diet 225 was designed to furnish as much protein, vitamins and salts per calorie as the diets of moderate or low fat content. Body weight gains were virtually the same on diet 225 and diet 226. Another high fat diet,

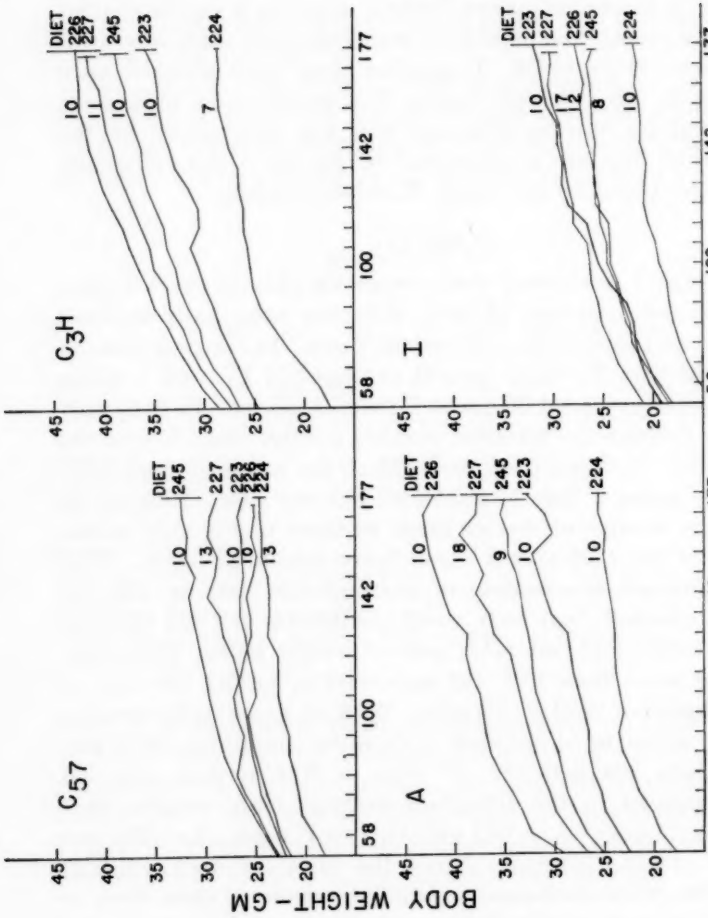

Fig. 1 Growth curves of 4 strains of mice. The numbers ranging from 7 to 18 near the ends of the curves indicate the numbers of animals in each group. The vertical lines at the ends of the curves show the magnitude of the standard errors.

257, was designed to simulate the composition of rat milk, at least as far as the proportions of protein, fat and carbohydrate were concerned. It was tried only with C_{57} and I strain mice and supported weight gains equal to, but not exceeding, those obtained on diet 245.

Since the high fat diet, 226, permitted the most consistent development of obesity in C_3H and A strain mice, it was

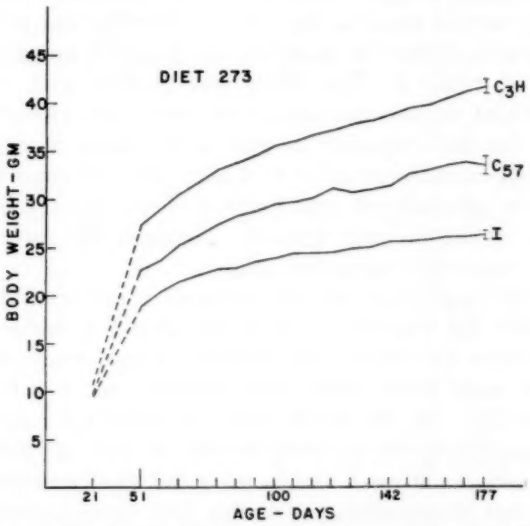

Fig. 2 Comparison of weight gains of three strains on 50% fat diet. The numbers of animals represented by the three curves are as follows: C_3H — 49; C_{57} — 19; I — 32. Standard errors are indicated for the weight averages at 177 days.

decided to design a new, somewhat simpler, high fat diet, no. 273, for more extensive work. The weight gains of three of our strains on this diet are shown in figure 2. It is again evident that the feeding of a high fat diet supported very substantial weight gains for the C_3H mice, while the final body weights of C_{57} and I strain mice were well below 35 gm.

Although the C_3H and A strain mice showed similar susceptibility to obesity, and C_{57} and I strain animals showed

similar resistance, it was decided not to combine the results obtained with C_3H and A strain mice on the one hand and those of the C_{57} and I strain animals on the other. There seemed to be sufficient differences among strains in their response to specific diets to justify reporting the results with each of the 4 strains. Much more information is needed on the inheritance of metabolic patterns.

Since body weight alone is not an adequate criterion of obesity, carcass analysis for fat and protein was carried out. The results of the fat analyses on A and I strain mice are shown in table 2. The analytical results with C_3H mice showed that the composition of the carcasses varied with the diet in the same manner as that of A strain mice. For this reason the results with the C_3H mice are not reported in the table. In general, the fat content of C_{57} mice followed the pattern of the I strain animals, although the fat content of the C_{57} mice was somewhat higher.

Several important aspects of table 2 are worthy of mention. The fat content of A strain mice fed the commercial stock ration was 11.9% at 6 months of age. Only when these animals were much older (14 months) did the fat content reach 19.3%. On the other hand, A strain mice fed diet 226 contained 25.2% fat at three months of age. No fat analyses were carried out on I strain mice fed the commercial stock ration, but observation has shown that these animals remain lean until fairly late in life. When mice of this strain were fed diet 227, the carcass fat reached a level of 16.6% at 6 months of age and 20.3% several months later. The table also bears out again the fact that this strain responds rather poorly to the high fat diet, no. 226. The greatest fat content of any individual animal was 46.8%, attained by a C_3H mouse.

Consideration of the data on carcass fat does not furnish a simple criterion for evaluating the degree of obesity. The question still remains of where to draw the dividing line between obese and non-obese animals. Figure 3 shows the carcass fat content plotted against fat-free weight for 215 animals regardless of age or dietary regime. Thus, some

TABLE 2

Relation of age and diet to fat content of carcass

	DIET NO.	AGE IN MONTHS									
		2			3			6			8+		
		BW [1]		*Fat* [2]	*BW*		*Fat*	*BW*		*Fat*	*BW*		*Fat*
A STRAIN	LC [3]	15.0	(3) [4]	8.5	...		...	27.4	(3)	11.9	33.5	(10)	19.3
	224	...		..	...		...	24.8	(3)	11.0	26.9	(3)	14.7
	232	...		..	...		...	27.9	(3)	12.2	...		...
	223	...		..	...		...	34.8	(3)	28.2	...		...
	227	...		..	34.9	(3)	24.6	39.6	(4)	29.3	36.4	(3)	22.1
	245	...		..	...		...	38.8	(3)	22.8	...		...
	226	...		..	37.1	(3)	25.2	37.8	(3)	26.8	...		...
I STRAIN	224	11.1	(5)	5.7	...		...	20.9	(3)	7.2	...		...
	232	...		..	23.0	(3)	5.8	24.0	(3)	8.5	...		...
	223	18.6	(5)	5.3	...		...	28.9	(5)	17.8	...		...
	227	16.9	(4)	6.0	28.9	(3)	13.1	31.6	(3)	16.6	31.7	(3)	20.3
	245	...		..	25.1	(3)	7.7	25.7	(6)	6.4	29.8	(3)	14.9
	226	19.3	(5)	7.0	24.6	(6)	8.7	26.2	(3)	9.4	...		...
	273	...		..	...		...	26.8	(3)	9.4	...		...

[1] Body weight, grams.

[2] Carcass fat in per cent of total body weight.

[3] Commercial stock ration.

[4] Number of animals.

of the data were obtained from animals only two months old, while some were from mice 16 months old. Some animals analyzed had been reared on the commercial stock ration, while the others had been fed the various experimental rations described in table 1. Since nearly half of the analyses were carried out on mice of the I strain, detailed calculations were possible only for the data obtained from these animals. The points plotted clearly fell along two regression lines. The

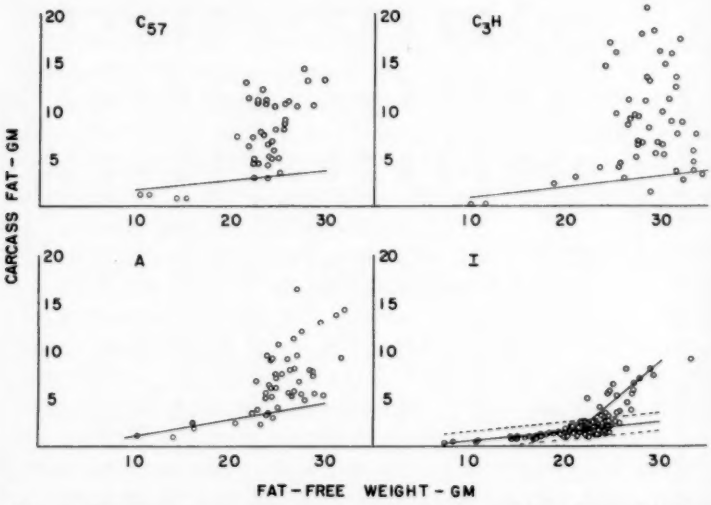

Fig. 3 Relation of carcass fat content to fat-free weight. Regression lines were calculated only for the results with I strain mice. The broken lines indicate 2 × standard error of estimate.

lean, small animals showed a slope of 0.1, while a few mice fell on a regression line of slope 0.8. The broken lines in figure 3 (section on I strain mice) indicate the limits of two times the standard error of estimate for the data on lean animals (slope 0.1). The results obtained with the other three strains fell into the same pattern.

In two experiments an effort was made to increase the caloric density of the diet at regular intervals. In the first

of these experiments, mice were placed on diet 227, containing 15% fat. After two weeks on this ration they were transferred to diet 245, containing 25% fat. After two more weeks they were fed diet 226, containing 47.5% fat. In the second of these experiments the animals were placed on diet 254, containing 10% roughage. After two weeks on this ration they were placed on diet 227, two weeks later on diet 245, and two weeks after that on diet 226. This periodic increase in the caloric density of the ration did not result in greater weight gain than would be expected from feeding diet 226 alone.

DISCUSSION

The data presented in this paper show that it was relatively easy to cause excessive deposition of fat in young mice of the C_3H and A strains by feeding complete, highly purified diets containing 30% casein and from 5% to 50% fat. On the other hand, it was difficult to accomplish the same end with C_{57} and I strain mice. The rate of fat deposition in C_3H and A strain mice was roughly proportional to the concentration of dietary fat. Deuel et al. ('47) and others have reported that the rate of weight gain of the rat increased with the dietary fat level. It seems, therefore, of some interest that mice of the C_{57} and I strains showed their best weight gains when fed diets of moderate fat content, and were actually inhibited by high fat diets.

Since we have already shown (Fenton and Carr, '51b) that C_3H and A strain mice consumed more food than the C_{57} and I animals, it will be of interest to feed some of our diets in isocaloric amounts. This has not been done in the present study because we were primarily interested in giving the animals unlimited access to food in order to permit the full expression of any inherited tendency to obesity.

The weight gain responses reported here have made it possible to investigate the relation of obesity to some aspects of carbohydrate metabolism, since we are now in a position to select for experiment (a) mice of the same strain kept

lean or made obese by feeding different diets, or (b) lean or obese mice of different strains fed the same diet. Marked differences between lean and obese animals have been observed with respect to glucose tolerance and glycogen deposition in the liver and isolated diaphragm.

Work reported here, or still in progress, indicates that metabolic patterns and probably endocrine balances are in part genetically determined. The inheritance of metabolic patterns has been demonstrated before. Danforth ('27) showed that obesity is associated with yellow coat color. Continued inbreeding of yellow mice leads to the disappearance of the tendency to obesity, but Fenton and Chase ('51) have shown that the feeding of a high fat diet again made possible the appearance of obesity in the yellow offspring, while the non-yellow litter mates did not become obese. Palmer and his associates ('46) developed two strains of rats showing different efficiencies of food utilization as well as other metabolic differences. Fenton et al. ('47, '50) have shown the existence of inherited patterns of requirements for riboflavin and pantothenic acid.

The growth inhibition observed when feeding high protein diets has been demonstrated by Bosshardt et al. ('46) and others. In the present long-term study, mice fed a 90% protein diet eventually attained reasonable body weights but deposited less fat than any other dietary group. If it could be shown that such high protein diets are not deleterious, they might then constitute a method of preventing excessive fat deposition.

It was hoped at the outset of this study that mice might become accustomed to consuming a certain bulk of diet. If this had been realized, the progressive increase in the caloric density of the diet should have caused rather rapid accumulation of body fat. This hope did not materialize under the experimental conditions used.

While it is relatively easy to recognize extreme obesity in the experimental mouse, it is always difficult to decide on adequate criteria for drawing the line between the obese and

non-obese. One can, of course, decide arbitrarily that all mice above a certain body weight shall be considered obese. Our data show, however, that even extreme deposition of fat was accompanied by accretion of protein and other non-lipid materials.

Drawing the dividing line between obese and non-obese on the basis of carcass fat content is perhaps more satisfactory. Such a dividing line is again rather arbitrary. The data plotted in figure 3 offer perhaps some rational basis for deciding whether or not an animal is obese. Considering for the moment the data obtained with I strain animals, two regression lines have been computed to express the available data. The majority of mice fell along the line of slope 0.1, but a few animals fell about a line of slope 0.8. (We recognize the possibility that the data might also be represented by a single exponential curve.) The data for the other three strains apparently can also be similarly expressed. Although relatively few small, lean animals of the C_{57}, C_3H and A strains were analyzed, the curves relating carcass fat to the fat-free weight of lean animals would certainly not differ strikingly from the curve for lean I strain mice, of which 71 were analyzed. It must be pointed out that the obese C_{57} and I strain mice plotted in figure 3 were considerably older than 6 months, and thus do not negate the earlier contention that these two strains do not become obese during the first 6 months of life.

The relationship between fat and fat-free weight indicated in figure 3 should, if it holds true generally, make it possible to decide more objectively between obese and non-obese animals. It would be necessary in each case to determine the linear relationship between carcass fat and fat-free weight for a number of obviously lean animals. Other individuals which deviate from this line by more than two times the standard error of estimate should be called obese.

High concentrations of lipids were found in the livers of animals which became obese on the purified diets. On the other hand, mice which became obese late in life after having

been fed the commercial stock ration did not usually show fatty livers. This problem is still under investigation.

SUMMARY AND CONCLUSIONS

1. Mice of the C_3H and A strains were made obese prior to 6 months of age by being fed certain highly purified diets. Animals of the C_{57} and I strains did not so respond.

2. The feeding of a high protein diet prevented the development of obesity.

3. Mice rendered obese by these nutritional means showed high levels of liver lipids.

4. The carcass fat content of lean animals increased linearly with the fat-free weight. It is suggested that an animal can be considered obese if it deviates from this linear relationship by more than two times the standard error of estimate of the regression of carcass fat on fat-free weight.

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STUDIES TO DETERMINE THE NATURE OF THE DAMAGE TO THE NUTRITIVE VALUE OF SOME VEGETABLE OILS FROM HEAT TREATMENT [1, 2]

III. THE SEGREGATION OF TOXIC AND NON-TOXIC MATERIAL FROM THE ESTERS OF HEAT-POLYMERIZED LINSEED OIL BY DISTILLATION AND BY UREA ADDUCT FORMATION

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ONE FIGURE

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INTRODUCTION

The deleterious effects of heating certain vegetable oils at polymerization temperatures on their nutritional value have been described in a previous paper (Crampton et al., '51c). There is some evidence (Crampton et al., '51a) that these deleterious effects are not a consequence of peroxidation. It has also been shown (Crampton et al., '51b) that the deleterious effects may be demonstrated by feeding the ethyl esters of heated linseed oil.

When the whole esters of heated linseed oil were separated by distillation at pressures of the order of 0.5 mm into a distillable fraction and a non-distillable fraction, then the non-distillable fraction was deleterious to experimental ani-

[1] Contribution from the Faculty of Agriculture, McGill University, Macdonald College, Quebec, Canada, Journal Series No. 304.

[2] Issued as paper 277, Canadian Committee on Food Preservation.

mals. Rats receiving this fraction at the level of 20% of the diet failed to grow; had oily, matted coats; excreted dark, sticky feces; and displayed a heavy mortality. Rats receiving the distillable fraction survived the experiment in good condition and excreted well-formed feces. However, they did not grow as well as rats receiving the whole esters of unheated linseed oil. In fact, their growth was no better than that of rats receiving the whole esters of heated oil. At this stage it seemed possible that the major defect of the heated oil arose from the presence of dimeric acid radicals. However, the distillable monomeric ester fraction was also nutritionally inferior to the esters of whole unheated linseed oil. This fact could not be related to differences in susceptibility to peroxidation, for the esters of the whole unheated oil were considerably more susceptible to peroxidation than the distillable esters of the heated oil (unpublished results).

It seemed possible, therefore, that the monomeric ester fraction might contain material other than straight chain esters, and that such material might play a part in determining the deleterious nature of the heated oils.

The formation of such materials by the exposure of the oils to polymerization temperatures carries implications of some importance so far as oils intended for food are concerned. Accordingly, the preliminary work was repeated and extended, and this paper deals with these later experiments.

EXPERIMENTAL

The nutritional test was performed in two replicates, each comprising observations on 60 animals. Six diets were tested. These diets differed only in (a) the nature of the oil component and (b) the proportion of white flour as adjusted to compensate for the use of 10 or 20% levels of oil.

The formula for the diets, in per cent, was as follows: white flour 53.65 or 43.65; skim milk powder 19; casein 11.5; oil preparation 10 or 20; brewers' dried yeast 3; feeding bone-

meal 2; iodized salt 0.5; chromium sesquioxide [3] 0.25; and ferric citrate 0.1.

The oil preparations were certain fractions of the ethyl esters of heat-polymerized linseed oil (12 hours at 275°C. in a strong current of carbon dioxide). The preparation of these fractions is described in detail in the Appendix. They will be designated in this paper for convenience as follows:

1. *Straight chain monomers from whole oil:* that portion of the ethyl esters of heated linseed oil which formed urea adducts with ease.
2. *"Cyclized" monomers and dimers from whole oil:* that portion of the esters of heated oil which did *not* form urea adducts.
3. *Straight chain plus "cyclized" monomers from distillate:* that portion of the esters of heated linseed oil which was distillable at pressures around 0.5 mm.
4. *Straight chain monomers from distillate:* that portion of the distillable esters which formed urea adducts.
5. *"Cyclized" monomers from distillate:* that portion of the distillable esters which did *not* form urea adducts.
6. *Dimers by distillation:* the residue after removal of the monomeric esters by distillation.

The probable general chemical relationships of these 6 fractions are conveniently indicated by a flow sheet (fig. 1). It is to be understood that the designations of the fractions and the symbols used in the flow sheet do not imply that the nature of the fractions is established. While it is reasonably certain that fraction 6 consists mainly of esters of dimeric acids (see table 5) it cannot be considered as finally established whether these are linked by single carbon-to-carbon bonds or by a cyclohexene system. Again, whether the non-adduct-forming monomeric material of fractions 2, 3 and 5 is cyclized or branched is not established: the term "cyclized" must be read as denoting a mode of preparation and not as asserting the presence of a cyclic structure. The chemical characteristics of the fractions are reported in table 5: the

[3] For determination of digestibility.

mean molecular weights confirm the validity of the designations so far as the terms monomeric and dimeric are concerned, but do not provide evidence as to the presence or otherwise of cyclic structures. It may be recalled, however, that Paschke and Wheeler ('49) suggested the presence of cyclic monomers in heat-polymerized methyl linoleate.

The hydroxyl contents of the fractions were determined by the method of Ogg, Porter and Willits ('45). The data

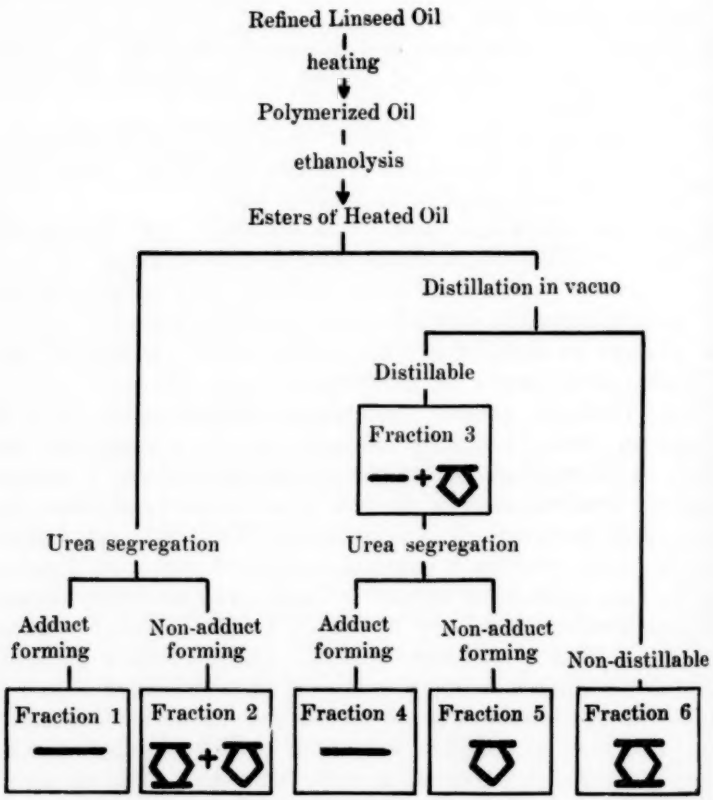

Fig. 1 Flow sheet illustrating preparation of fractions of esters of heated linseed oil used in feeding trials. (For details of preparation, see Appendix.)

are reported in table 5. So far as the monomeric fractions 1, 4 and 5 are concerned, the values correspond to not more than 8, 5 and 15%, respectively, of monohydroxy C18 acids. The available evidence therefore supports the view that hydroxy acids did not constitute a major proportion of the non-adduct-forming monomeric material.

The feeding trials lasted 28 days. Live weight changes and feed consumption were recorded. The apparent digestibilities were determined by the Cr_2O_3 index technique (Schürch et al., '50). Digestible calories were calculated from the gross calorific values of the diets, the feed intake and the simultaneously determined digestibility coefficients. Live

TABLE 1

Comparison of the effect of 10% versus 20% of fractions of the esters of heat-polymerized vegetable oil in the diet of rats (60 rats at each level)

OIL IN DIET	RATS SURVIVING 28 DAYS OF TEST	AVERAGE GAIN IN 28 DAYS	AVERAGE FEED IN 28 DAYS	DIGESTIBILITY OF FEED	GAIN ADJUSTED TO EQUAL INTAKE OF DIGEST. CALORIES
%	%	*gm*	*gm*	%	*gm*
10	83	73	262	91	67
20	63	27	177	83	52
Least sig. diff. (P = 0.05)	..	11	17	..	8

weight gains were adjusted by simple regression to equal intake of digestible calories to provide a measure of the relative efficiency of the diets. Least significant differences between means, where shown, were calculated from the expression

$$\text{L.S.D.} = \frac{\sigma}{\sqrt{n}} \times \sqrt{2} \times t_{5\%}.$$

In the preliminary tests, the use of diets containing 20% of certain ester fractions similar to those used in the present experiments had resulted in heavy mortality. Consequently, half of the rats in each of the lots in the tests now reported received a diet containing 10% oil. The differences between the responses of the rats receiving these two levels of oil are summarized in table 1.

Efficacy of protection of the fractions and diets against autoxidation

Since peroxidized fats and fatty acid esters may be toxic to rats, it is necessary to ensure that the results of feeding such materials are not vitiated by the effects of peroxidation. It has been shown that the deleterious effects of heated linseed oil are not related to the degree of susceptibility of the heated oils or of the diets containing the oils to peroxidation (Crampton et al., '51a). In the present experiments the fractions used in the diets were prepared immediately

TABLE 2

Peroxide values of oils extracted from the mixed feeds at two weeks and 4 weeks after preparation. Feeds stored in refrigerator

FRACTION CONTAINED IN THE DIET	PEROXIDE VALUE, MG PEROXIDE OXYGEN PER KG OIL	
	After 2 weeks	After 4 weeks
Fraction 1	44	98
Fraction 2	38	88
Fraction 3	42	90
Fraction 4	46	102
Fraction 5	38	92
Fraction 6	33	75

before feeding, and were protected by addition of an antioxidant and storage in the refrigerator in the dark. The stability of the oils in the diets was tested at the end of the second week of the second replicate and again at the end of the 4th week. Peroxide values of the lipid components of the feed were determined by the method of Skellon and Wills ('48) as described by Crampton et al. ('51a). Peroxidation was relatively slight in all fractions (table 2). From unpublished observations it is believed that peroxidation would not have begun to interfere with the nutritional well-being of the rats until the peroxide value of the oil extracted from the feed attained values of the order of 1,000 mg per kilogram of oil.

It may be concluded, therefore, that the results of the present feeding experiments were due to the effects of constituents other than products of peroxidation.

RESULTS

With diets containing 20% oil

The results of feeding these 6 oil fractions in diets in which they constituted 20% by weight are summarized in table 3. The first salient feature of the results relates to the *mortality*. It is clear that the presence of the "cyclized" monomers in the diet was associated with toxicity, though the mode of action of this material remains obscure. Rats consuming fraction 5, which contained the highest concentration of this material, survived only 5 to 10 days in spite of a greatly reduced feed intake (and hence low intake of toxic material).

A comparison of the results for diet 2, containing a mixture of approximately three parts dimeric esters to one part "cyclized" monomer, with the results for diet 6, shows clearly the much more harmful nature of the monomeric material. The deaths in group 6 were associated with partial starvation and excessive diarrhoea, whereas the animals of group 2 displayed neither diarrhoea, digestive disturbances, nor overt clinical symptoms suggestive of the cause of death.

The animals receiving fractions consisting mainly of straight chain monomers — i.e., groups 1 and 4 — all survived the test and remained in good health. Their gains were comparable to those secured in previous tests with esters of whole unheated linseed oil.

The *gains* of the rats receiving the various fractions also reflected the toxic effects of the "cyclized" monomeric material and the adverse effects of the dimeric esters. The dimeric ester fraction was associated with low digestibility, and this was doubtless the cause of the diarrhetic condition of the rats receiving this material.

The *relative efficiencies* of the diets *per unit of digestible calories eaten* showed that the straight chain monomeric

TABLE 3

The effect of feeding fractions of polymerized linseed oil at 20% level in rat diets (10 rats per lot)

OIL FRACTION FED	PRINCIPAL OIL CONSTITUENTS	NO. OF ANIMALS SURVIVING TEST	AVERAGE GAIN	AVERAGE FEED INTAKE (dry matter)	DIG. OF DRY MATTER	DIG. OF FAT FRACTION	MEAN GAINS ADJUSTED TO EQUAL INTAKE OF DIG. CAL.
			gm	*gm*	*%*	*%*	*gm*
1	Straight chain monomers of whole oil	10	101	266	93	94	72
2	"Cyclized" monomers plus dimers of whole oil	2	—32	141	81	77	4
3	Straight chain plus "cyclized" monomers of distillate	9	4	118	94	97	52
4	Straight chain monomers of distillate	10	57	193	94	95	63
5	"Cyclized" monomers of distillate	0	..	..	..	..	..
6	Dimers	7	13	168	55	29	71

esters and the dimeric esters were equally well "metabolized," whereas the presence of "cyclized" monomeric material resulted in a sharp reduction in the utilization of digestible calories.

The *low feed intake* by group 5 made it possible that this might have caused the death of some of the rats receiving the "cyclized" monomers. This point was examined by feeding a supplementary group of 5 rats the basal oil-free diet ad libitum. They were dosed daily by dropper with a quantity of "cyclized" monomer equal to 10% by weight of their measured voluntary intake of the fat-free mixture. The intake of the basal diet under these conditions was low, but it was adequate for maintenance and in one case permitted some gain. Nonetheless, all of these rats died within 16 days, thus confirming that the mortality in group 5 could not be explained on the grounds of low food intake.

It will be noted from the schematic outline (fig. 1) of the preparation of the fractions that fractions 1 and 4 ought to consist mainly of the straight chain adduct-forming material. However, fraction 1 had a lower iodine number and a lower refractive index than fraction 4 (table 5). Moreover, the yield of fraction 1 represented 46%, whereas that of fraction 4 was 49%, of the total esters. These differences in yield and in the characteristics of presumptive straight chain monomeric material according to the route by which it has been prepared, suggest that in the case of fraction 4 the adduct-forming fraction had probably carried with it by entrainment a small proportion of material incapable of itself of forming an adduct; i.e., of "cyclized" monomer.

Fraction 1 was also superior to fraction 4 in nutritional value. This circumstance reinforces the view that fraction 4 contained a small proportion of the non-adduct-forming material ("cyclized" monomer), and that the "cyclized" monomeric ester is either of itself the deleterious component or that the deleterious material accompanies this ester fraction.

TABLE 4

The effect of feeding fractions of polymerized linseed oil at 10% level in rat diets (10 rats per lot)

OIL FRACTION FED	PRINCIPAL OIL CONSTITUENTS	NO. OF ANIMALS SURVIVING TEST	AVERAGE GAIN	AVERAGE FEED INTAKE (dry matter)	DIG. OF DRY MATTER	DIG. OF FAT FRACTION	MEAN GAINS ADJUSTED TO EQUAL INTAKE OF DIG. CAL.
			gm	*gm*	%	%	*gm*
1	Straight chain monomers of whole oil	10	107	300	95	94	72
2	"Cyclized" monomers plus dimers of whole oil	10	39	205	87	73	58
3	Straight chain plus "cyclized" monomers of distillate	10	66	228	94	96	66
4	Straight chain monomers of distillate	10	108	299	94	95	75
5	"Cyclized" monomers of distillate	0	..	..	91	93	..
6	Dimers	10	77	276	87	69	64
Least significant difference (P = 0.05)		..	23	36	..	..	16

With diets containing 10% fat

The results of feeding the diets in which the fat levels were reduced to 10% are shown in table 4. They were in all respects what would have been predicted from the results of feeding the 20% fat levels. The toxic effect of the "cyclized" monomers had been lessened, though it was still clearly in evidence. Gains, feed intake, digestibility and efficiency of utilization from the straight chain monomers were practically identical as between rats in lot 1 with 20% fat and those of lots 1 and 5 with 10% fat. Even at this lower fat level none of the rats receiving the "cyclized" monomeric material (lot 5) survived 28 days; where this "cyclized" monomeric material constituted only 2.5% of the diet intake (lots 2 and 3), the rats were able to complete the test, but with significantly smaller gains.

CONCLUSIONS

1. Heat polymerization of alkali-refined linseed oil results in the formation of monomeric acyl radicals whose esters can be distilled at low pressures but which do not form urea adducts. These esters are highly digestible when eaten but are not utilized for growth. The esters contain a small proportion of hydroxyl groups, but are apparently mainly esters of "cyclized" or branched structure.

2. When a fraction consisting essentially of these esters of "cyclized" monomeric acids comprises as much as 10% of the diet, it renders the diet "toxic." The harmful effect is measurable when this fraction comprises 2.5% of the diet. The effect is ascribed tentatively to the "cyclized" monomeric esters as such, rather than to the small proportion of hydroxy acid present in this and other fractions.

3. Dimeric acyl radicals are also formed on heating linseed oil at polymerization temperatures. A fraction consisting mainly of the esters of the dimeric acids is non-toxic, but this material is largely unabsorbed from the gastrointestinal tract. Its presence results in excreta of the nature and consistency of a mixture of soft feces and fresh varnish.

4. Esters of straight chain monomeric acids (i.e., capable of forming urea adducts) are apparently as nutritious as are esters prepared directly from normal unheated triglyceride oils which, in turn, are but slightly inferior to the triglycerides of normal food.

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APPENDIX

Preparation of ethyl esters of polymerized linseed oil

Commercial alkali-refined linseed oil (Canada Linseed Oil Mills, Montreal, P.Q.) was heated in a stream of CO_2 for 12 hours at 275°C. The oil was heated in batches of about 1,200 gm at a time, and the CO_2 was passed at a rate sufficient to keep the oil surging fairly freely. The resultant polymerized oil was of a clear light straw color.

The polymerized oil was allowed to cool while maintaining the current of CO_2. The polymerized oil was then shaken up with one-third of its volume of absolute ethanol to remove any fatty acids present. The washings were discarded.

The washed polymerized oil was used for the preparation of the ethyl esters by alcoholysis, using caustic soda at the rate of 0.5% by weight of the oil as catalyst (Feuge and Gros, '49). The esters were washed with large volumes of hot distilled water in a large separatory funnel. The total yield of mixed esters reckoned on the weight of oil taken was approximately 90% (varying from 88 to 92 with different batches).

Fractionation of the ethyl esters for the feeding trials

A portion of the total ethyl esters was subjected to vacuum distillation as described by Crampton et al. ('51b). The non-distillable fraction, comprising the ethyl esters of dimeric fatty acids, constituted fraction 6. The distillable portion, comprising the ethyl esters of monomeric acids, constituted fraction 3.

Immediately after distillation Tenox II * (Tennessee Eastman Corp.) was added to the portions of fractions 3 and 6 reserved for feeding at the rate of 0.1%. The protected ester fractions were at once removed to the cold room (5°C.), and held there until incorporated in the diets.

To another portion of fraction 3, Tenox II was added at the rate of 0.025%. This portion was subjected to fractionation by urea adduct formation (Schlenk, '49). The following technique was used.

Five hundred grams of the esters were dissolved in 2 l absolute commercial ethanol in a 4-l beaker. Solid urea was added slowly with stirring, while maintaining the mixture at 50°C. In all 2 kg urea were added, since approximately 3 gm urea are required for each gram of C18 ester for adduct formation. The mixture was maintained at 50°C. for about 30 minutes and then allowed to stand at room temperature overnight. Next morning the adduct and excess urea were filtered off and washed on the Buchner funnel with absolute ethanol saturated with urea. The adduct was decomposed by being thrown into warm water. Immediately the oily layer separated, Tenox II was added at the rate of approximately 0.25% of the adduct-forming material. The oily material was washed with water and dried under high vacuum with shaking. This material constituted fraction 4, and presumably consisted mainly of straight chain monomeric esters (see table 5). The non-adduct-forming material in the supernatant and ethanolic washings was separated by the addition of copious amounts of water. No Tenox

* Tenox II is a preparation containing 20% butylated hydroxyanisole, 4% citric acid, 6% propyl gallate and 70% propylene glycol.

II was added, since presumably that present before adduct formation would tend to be concentrated in this fraction, which constituted fraction 5. The addition of small quantities of sodium chloride facilitated the separation of both fractions 4 and 5 from the aqueous phase. Fraction 5 was dried in the same fashion as was fraction 4. Both fractions were now further protected against autoxidation by the further addition of 0.1% Tenox II and stored under the same conditions as fractions 3 and 6.

Another portion of the total esters was separated into adduct-forming (fraction 1) and non-adduct-forming fractions (fraction 2) by the technique described above, except that the total weights of the esters and ethanol were correspondingly reduced, since the total esters of heated oil contained a much smaller proportion of straight chain monomeric material than did the distillable esters (fraction 3). The same precautions were taken against peroxidation as when preparing fractions 4 and 5.

The analytical characteristics of the fractions are presented in table 5.

TABLE 5

Yields and characteristics of fractions of ethyl esters used in the feeding trials

	FRACTION NUMBER	YIELD AS % TOTAL ESTERS OF HEATED LINSEED OIL	IODINE NO.[1]	REFRACTIVE INDEX n_D^{25}	MEAN MOLECULAR WEIGHT	HYDROXYL[2] VALUE AS PER CENT (— OH)
1	Adduct-forming fraction of total esters	46	118.2	1.45345	293	0.23
2	Non-adduct-forming fraction of total esters	54	162.7	1.47561	472	0.46
3	Distillable esters	60	130.1	1.45684	294	0.22
4	Adduct-forming fraction of distillable esters	49	124.8	1.45494	293	0.14
5	Non-adduct-forming fraction of distillable esters	11	170.7	1.46986	300	0.39
6	Non-distillable esters	40	159.9	1.48017	550	0.64

[1] Iodine numbers by the method of Benham and Klee ('50), using one-hour reaction time (Klee and Benham, '50).

[2] Hydroxyl values by the method of Ogg, Porter and Willits ('45).

EFFECTS OF PROLONGED INGESTION OF XYLOSE ON RATS

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Xylose is a pentose sugar which might conceivably be incorporated into foods for human consumption. In terms of sweetness it has a value of 40 when compared to sucrose at 100. Monogastric animals are unable to utilize this 5-carbon sugar (Pflüger, '05; Miller and Lewis, '32; Anderson, '50), which suggests that it might be used as a dietary sweetening agent by individuals who desire to reduce body weight. Xylose might also serve as a sweetening agent for diabetics, either in addition to or in lieu of saccharin. The tendency of this sugar to cause diarrhea in laboratory animals suggests its possible use in laxative preparations. In the event a use is found for xylose, it could be produced in quantity from cottonseed hulls and corn cobs, thanks to the methods of production developed by Dunning and Lathrop ('45).

The possible inclusion of xylose in foods immediately raises the question as to whether any health hazard would be involved. The literature concerning the utilization of this substance by man is neither very extensive nor conclusive, but some experimental work has been done using laboratory animals. Darby and Day ('39) reported on the cataractogenic action of xylose in rats. These same workers (Darby and Day, '40) and others (Anderson, '50; Blatherwick et al., '36) observed diarrhea and abdominal distension when rats received this sugar orally. Blatherwick and co-workers ('36) fed

rats a diet containing 5% xylose for a period of 64 days. In addition to the diarrhea, they found albuminous degeneration of the liver cells, but these effects were not permanent.

The performance of rats on various levels of intake and on different basal rations for long periods of time has not as yet been adequately studied. The present work was undertaken to get some indication of how much xylose rats can ingest continuously without encountering any adverse effects.

EXPERIMENTAL

The xylose [1] was fed to rats as a supplement to three different basal diets. One was identical to, or a modification of, that used by Darby and Day ('39) and consisted (in percentage) of crude casein 18, salts 3, cod liver oil 2, butterfat 6, yeast 10, cornstarch 26, and monosaccharide 35. The monosaccharide consisted of xylose adjusted with glucose to total 35. A commercial laboratory ration [2] was also used, the added xylose being substituted at the expense of the entire ration. A third basic diet was primarily a mixture of natural feeds, and is the diet most commonly used in this laboratory. It is described in a previous report (Wilson and DeEds, '50) as the Addis diet. Obviously these latter two feed mixtures would be somewhat comparable to human diets.

Unless otherwise indicated, the rats were weanling Sprague-Dawley females. They were housed in individual wire-bottom cages and received tap water and the particular diet ad libitum. A weekly record of body weights and food consumption was maintained. Five rats were used per group.

Ophthalmoscopic examinations of the eyes were made during the entire experimental period at weekly intervals. Before examination the iris was dilated by instillation of a drop of 0.5% atropine sulfate solution into each eye.

[1] Obtained from the Northern Regional Research Laboratory, Peoria, Illinois. It gave the following analysis: $[\alpha]_D^{25} = +19.5°$; ash 0.20 to 0.37%.

[2] Purina laboratory chow. (Mention of this product does not imply that it is endorsed or recommended by the Department of Agriculture over others of a similar nature not mentioned.)

All rats surviving the 20-week feeding period were autopsied and examined grossly, and tissues were saved for histological study.

Diabetic rats were obtained by injecting alloxan. Various dosage levels were tried as well as subcutaneous, intraperitoneal, and intra-cardiac routes of administration. The criterion for selection of diabetic rats was the presence of elevated blood sugar levels, determined by the method of Folin and Malmros ('29). Polyuria was another evidence of diabetes. The blood sugar levels were used as a basis for dividing the diabetic rats into two comparable groups, one being fed the Addis basal and the other receiving the Addis basal plus 15% xylose ad libitum. By so doing, the influence of the level of blood sugar on the extent of the lens opacity was equalized.

RESULTS

Using albino rats from our own colony (Wilson, DeEds and Cox, '41), it was observed that 21-day-old rats developed severe lens opacities in less than 10 days after receiving an Addis or Darby and Day basal ration containing 35% xylose. Comparable rats placed on these same diets, but at 35 to 40 days of age, had failed to develop any lens opacities after 5 weeks. Mitchell ('36) encountered similar results when feeding rats lactose or galactose, and demonstrated that age and strain have a marked effect on the susceptibility to cataracts.

In table 1 the results of two separate experiments involving the prolonged feeding of xylose to weanling rats have been consolidated. In the last column, titled "Ophthalmoscopic observation," it can be seen that no lens opacities were noted until the level of xylose constituted at least 15% of the diet. This appeared to be the critical level, regardless of which of the three basic diets was used. As the level was raised above 15%, there was a proportionate increase in the severity of the opacities observed. An arbitrary scale was established to indicate the degree of opacity as slight, moderate, severe or complete, depending on whether the area of opacity rep-

resented one-fourth, one-half, three-fourths or the entire lens area. Without exception the opacity was first observed in the periphery of the lens as a diffuse cloudiness, which then became more dense and progressed toward the center of the lens. When the intake of xylose was more than 20% of the diet, total lens opacity (no reflected light when the lens was examined with the ophthalmoscope) invariably resulted within three weeks.

A regression of lens opacity (recovery of lens transparency) was an unexpected observation in all but two rats. This regression was usually well underway within 60 days from the time of development of maximum opacity, but it should be emphasized that the regression was never complete. Thin circular vestigial lines of opacity, with transparent lens tissue both within and without the circle, were still visible with the ophthalmoscope even after 140 days, whether the rats were continuously receiving xylose in the diet or were taken off the experimental diet after only 21 days. Whether these thin dark circular lines were detrimental to normal vision is not known.[3]

Without exception diarrhea was noted within 48 hours after feeding this sugar at levels of 15% or more, but following a few weeks of adjustment the abnormal softness of the feces became less noticeable. The observation of bloat was suggestive of an abnormal fermentation in the digestive tract, due to xylose ingestion, but it was confined to the 25 and 35% groups and not all rats in a given group were affected.

From an inspection of table 1 concerning food intake and growth rates, it can be seen that group 13 (receiving 25% xylose plus the Addis diet) averaged only 207.6 gm in body weight, compared to group 2 (control) which weighed 254.4 gm after 20 weeks. This depression in growth was found to be highly significant when the individual body weights of the

[3] In the publication by Fitzhugh and Buschke ('49) there is a picture (fig. 3) of a rat lens (from an animal which had been fed β-tetralol) containing a dark circular line of opacity which strikingly resembled opacities which had regressed in our xylose-fed rats.

TABLE 1

Effect of xylose on food consumption, body weight, and lens transparency

	DIET	DAYS ON EXPT.	AVERAGE FOOD CONSUMPTION GM/RAT/DAY	FINAL AVERAGE BODY WEIGHT	OPHTHALMOSCOPIC OBSERVATION
1.	Darby and Day basal (DD)	140	10.6	213.4	No lens opacity.
2.	Addis basal	140	13.5	254.4	No lens opacity.
3.	DD + 5% xylose	140	10.8	223.8	No lens opacity.
4.	Addis + 5% xylose	140	12.5	246.8	No lens opacity.
5.	Purina + 5% xylose	140	15.3	226.6	No lens opacity.
6.	Addis + 10% xylose	140	13.8	226.4	No lens opacity.
7.	DD + 15% xylose	140	11.4	211.2	3 of 5 rats, moderate lens opacities, then regression.
8.	Addis + 15% xylose	140	12.6	224.2	2 of 5 rats, slight lens opacities, then regression.
9.	Addis + 15% xylose	140	13.1	210.4	2 of 5 rats, moderate lens opacities, then regression.
10.	Purina + 15% xylose	140	16.2	223.2	3 of 5 rats, slight lens opacities, then regression.
11.	Addis + 20% xylose	140	13.9	212.7	5 of 5 rats, severe lens opacities, then regression.
12.	DD + 25% xylose	21	...	....	5 of 5 rats, complete lens opacities, then regression.
13.	Addis + 25% xylose	140	13.4	207.6	10 of 10 rats, complete lens opacities, 8 of 10 regression.
14.	Purina + 25% xylose	21	...	...	5 of 5 rats, complete lens opacities, then regression.
15.	DD + 35% xylose	21	...	...	4 of 4 rats, complete lens opacities, then regression.
16.	Addis + 35% xylose	21	...	...	5 of 5 rats, complete lens opacities, then regression.

rats in these two groups were treated statistically. The food consumption for these two groups was almost the same; namely, 13.4 and 13.5 gm per rat per day, respectively. However, since added xylose is not metabolized for energy or growth, the actual or corrected net food intake would be 25% less than 13.4 gm, or 10.05. Conversely, in order for rats receiving 25% xylose to gain weight equal to that gained by the controls, the food intake would have to be 13.5/0.75, or 18 gm. It appears, then, that the intake of utilizable food was reduced (voluntary semi-starvation) as the per cent of xylose in the ration increased. This observation is in agreement with that of Anderson ('50).

Concerning survival rates, only two rats failed to survive the 140-day experimental regimen, out of a total of 60 animals. Since there were no deaths on the highest level of xylose (25%), it is concluded that the ingestion of xylose did not increase mortality.

At the end of the experiment all surviving rats were killed by ether and decapitation. Post-mortem examination for gross pathological changes revealed no abnormalities other than lung infections scattered throughout all groups. Sections of the following tissues were studied by Dr. A. J. Cox, Jr., Department of Pathology, Stanford University School of Medicine, San Francisco, California: thyroid, heart, lungs, liver, spleen, kidney, adrenal, intestine, pancreas and ovary. The only change directly related to the amount of xylose in the diet was seen in the liver. Livers from animals which received 25 or 35% xylose for 37 to 39 days showed definite, though slight, swelling of the cytoplasm of the hepatic cells. There was some separation of the stained particles within the cytoplasm but no definite vacuolation was seen in most of the cells. The nuclei appeared normal and there was no reason to conclude that the liver cells had been injured. The appearance was suggestive of a storage phenomenon. Livers from rats which received 15% xylose for 22 weeks also showed some similar swelling of the cytoplasm. This was not definite in the animals receiving 5% xylose.

As mentioned earlier, xylose may be ingested by diabetics. Medical records substantiate the fact that the incidence of lens opacities in diabetics is appreciably higher than for non-diabetics (Adler, '48). Would super-imposing of one caratactogenic agent upon another enhance the formation of cataracts? Attempts were made to feed xylose to rats made diabetic through the use of alloxan, according to the procedure described by Kass and Waisbren ('45). The yield of alloxan-diabetic rats was so low that out of 223 treated animals of different ages, sex, and strain, only 19 survivors were obtained with blood sugar at a level above 200 mg per 100 ml, of which 7 died before cataracts developed. Six diabetic rats, maintained on the Addis ration only, developed cataracts in approximately 41 to 57 days. However, 6 diabetic rats fed the same basal ration containing 15% xylose experienced cataracts as soon as the controls, but with a progressively much greater loss of lens transparency with respect to time. In fact, when the lens opacities were visible to the naked eye in the rats receiving xylose, the opacities in the controls were not noticeable. Greater numbers of animals will be required to verify these results, but the severity of the cataracts was decidedly in the direction of the xylose-fed rats.

DISCUSSION

The question is raised as to why xylose-fed weanling rats initially got rather severe lens opacities and then with time regained much of the lost transparency. There is no apparent answer to this question. One can only point out that the observed spontaneous reversibility of the lens opacity was in accord with the finding that in older rats the incidence of cataracts is either absent or greatly diminished when xylose is fed. Bellows and Chinn ('41) speculated that the basic cause of lens opacities was a non-specific osmotic derangement, either local or systemic. Such a mechanism would explain why a fairly large list of chemically unrelated compounds have been found capable of producing cataracts. These

same workers also concluded that the regression of experimental cataractous changes in the early stages points to a physical alteration which is reversible, rather than to irreversible chemical changes.

The results of the present study confirm the rather limited observations reported in the literature that xylose is toxic. If we can assume that humans would react in the same manner as rats, then xylose should not be incorporated into foods. It is true that the effects of xylose on the liver cells were reversible (Blatherwick et al., '36) and that a relatively high level of 15% in the diet was required to get detectable lens opacities in rats. More work would be required to determine whether it would be safe at lower levels when the ingestion was continued for longer periods. Again assuming that rats and humans would react in a comparable manner, it would seem that xylose might be more toxic for individuals with a tendency towards cataract (diabetics, for example) than for normal individuals.

SUMMARY

Xylose was fed for periods up to 140 days to rats at various levels from zero to 25% of the diet. Thirty-five per cent of xylose was fed for short periods. In weanling rats, a level of 15% or more was found to produce lens opacities, which regressed to a considerable degree with time whether or not the animals were continued on the experimental diet. The regression was never complete. Other toxic symptoms observed were diarrhea, bloating, and liver changes suggestive of storage. A voluntary restriction of utilizable food by the xylose-fed rats led to decreased rates of growth. Rats rendered diabetic with alloxan experienced more severe lens opacities when fed xylose than did diabetic controls. Pending more favorable experimental data at lower levels of intake, it is deemed inadvisable to risk the incorporation of xylose in foods at any level of intake for extended periods of time.

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THE VITAMIN A REQUIREMENT OF THE MOUSE[1]

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ONE FIGURE

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The minimum daily requirement of the rat for vitamin A has been the subject of many investigations. The results reported vary depending upon the criteria used and upon the methods of investigation employed. Paul and Paul ('46) recommended 10 I.U. of vitamin A per 100 gm of body weight. They based their recommendation upon the observation that this level represents the lowest level at which a maximum life span can be attained. Goss and Guilbert ('39) estimated the minimum vitamin A intake necessary for the prevention of deficiency symptoms at about 2 I.U. per 100 gm of body weight. As their criterion these authors used the minimum amount which would prevent the appearance of cornified vaginal smears. Sherman and co-workers ('45) found that vitamin A at a level of 12 I.U. per gram of food was optimum for rats. This amount represents about 4 times the level accepted as the minimum adequate for this species. The minimum levels, although adequate for growth and for complete protection against deficiency symptoms, failed to maintain the health of the animals as measured by successful reproduction, lactation, and the maintenance of body weights in the adult. These animals died at an early age in adult life as compared with rats receiving vitamin A at a level of 12 I.U. per gram of food daily.

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With respect to the mouse and its daily requirements for vitamin A, there have been no quantitative experiments reported to date. Rosenberg ('45) estimated the vitamin A requirement of the mouse, deriving his values from studies on other species, which he extrapolated to the mouse on the basis of comparative body weights. For the adult mouse he calculated the daily requirement to be 1 μg of β-carotene or 0.1 to 0.2 μg of vitamin A. This value does not allow for storage. For growth and reproduction he states that at least three times that amount of vitamin A is needed daily.

The purpose of the present study was to determine experimentally the minimum daily requirement of the mouse for vitamin A. Since it has been shown that absorption, utilization, and storage of vitamin A are influenced by the type of diet consumed and by the source of vitamin A employed, it seemed of interest to determine this requirement using a highly purified diet and a crystalline vitamin A preparation.

EXPERIMENTAL

The problem of ascertaining the minimum daily requirement of the mouse for vitamin A was investigated from two viewpoints; viz., from a curative approach and from a prophylactic one.

In curative studies, animals were depleted until the external symptoms of an avitaminosis were clearly established. Vitamin A was then administered in graded doses in order to determine the least amount of the vitamin which would restore and maintain the animals' health. A variation of this method consisted of calculating the minimum daily requirement from a determination of the time required to cause the reappearance of deficiency symptoms after the administration of known single doses of vitamin A to acutely depleted animals. According to the data obtained in these preliminary experiments, the minimum requirement essential to cure and prevent the reappearance of a pathological state varied between the limits of 0.75 I.U. to 3.0 I.U. daily. Inherent inadequacies of the curative approach with respect to vitamin A

studies consisted in an inability to control, or to estimate, the severity of the deficiency in the intact animal, the possibility of irreversible changes, and the deployment of an unknown percentage of the administered vitamin A for curative purposes, which would vary from animal to animal.

The results obtained utilizing the prophylactic approach will be reported in this paper. The procedure consisted of measuring selected physiological responses in a control group of mice consuming maximum quantities of vitamin A and of the other known dietary essentials. These physiological responses comprised the criteria against which were compared the responses of mice receiving vitamin A in graded doses. The lowest level of this vitamin which gave the maximum response — i.e., which prevented any depression in physiological responses — is considered the minimum daily requirement. The criteria chosen to determine the adequacy of the various levels of vitamin A supplementation included: (a) optimum growth rates and final weight gains, (b) the prevention of pathological effects due to an insufficiency of vitamin A, (c) mortality rates and longevity, (d) the maintenance of a normal reproductive function in the male mouse, and (e) the presence of a normal blood picture. The ability of the various levels to permit storage was also investigated.

In order to obtain a highly standardized group of animals depleted of vitamin A during the early stages of growth, a group of pregnant females of the Swiss-Webster strain was placed on a vitamin A-free diet at parturition. Litter sizes were cut down to an average of 6 young per litter, and only those young whose weights fell within a definite weight range were utilized. The young were placed at weaning on a vitamin A-free diet of the composition shown in table 1. The depletion period was discontinued when the animals were 36 days of age, or just prior to the appearance of the external symptoms of a deficiency state. This precaution minimized the possibility of irreversible changes due to vitamin A deficiency, and insured that an unknown fraction of the supplement administered would not be deployed for curative pur-

poses. It also permitted the administration of vitamin A supplements during the period of rapid growth. At the end of the 36-day depletion period, mice of both sexes were divided into 5 groups each in such a manner that the average weights of all groups were identical. The average weight of the males was 18.5 gm and that of the females was 16.7 gm. At the termination of the depletion period, 4 of the 5 groups received vitamin A in graded doses. Three of these groups were fed the basal vitamin A-free diet supplemented by the addition of 1, 2, or 3 I.U. of vitamin A per day. The 4th group received

TABLE 1

Experimental diet

INGREDIENTS	AMOUNT	SUPPLEMENTS ADDED PER KILOGRAM OF DIET	
	%		
Casein	20	Thiamine hydrochloride	20 mg
Sucrose	68	Riboflavin	20 mg
Salts [1]	5	Pyridoxine hydrochloride	20 mg
Ruffex	2	Calcium pantothenate	40 mg
Hydrogenated vegetable oil [2]	5	Folic acid	20 mg
		Choline chloride	500 mg
		alpha-Tocopherol	20 mg
		Vitamin D (Drisdol)	5,000 units

[1] Osborne-Mendel salt mixture.

[2] Crisco.

the basal diet to which had been added 67,500 I.U. of vitamin A per kilogram of diet, so that each mouse received approximately 210 to 300 I.U. per day, depending upon the food intake. A 5th unsupplemented group continued on the basal diet was used as negative controls.

The source of the vitamin A supplement employed in these studies was the U.S.P. reference standard, which is a solution of crystalline vitamin A acetate in cottonseed oil, suitably diluted for administration purposes.

RESULTS AND DISCUSSION

The beneficial effect exerted by the addition of vitamin A even at a level of 1 I.U. per day was noted as early as two

weeks after the initiation of supplementation, as is shown by the data in table 2. This effect was observed in two experiments using 68 and 52 animals, respectively. The greater susceptibility of the male to the onset of the eye condition was also noted in both experiments, indicating perhaps a more rapid depletion of the vitamin A stores, or a greater requirement on the part of the male mouse.

TABLE 2

Result of two weeks of vitamin A supplementation

GROUP	AVERAGE WEIGHT GAIN		INCIDENCE OF THE EYE CONDITION	
	Male	Female	Male	Female
	gm	*gm*	%	%
A-free	3.6	3.7	66	33
1 I.U.	5.0	4.6	0	0
2 I.U.	5.4	4.6	0	0
3 I.U.	5.8	4.8	0	0
Control	6.8	4.5	0	0

a. Growth rates

Typical growth curves for both males and females receiving the various levels of vitamin A supplementation are shown in figure 1. The growth rates of all animals receiving the vitamin A-free rations showed a marked drop after the 7th to 8th week of life. The beneficial effect of vitamin A is evident on all levels of supplementation. The growth curves of the supplemented mice are approximately the same for all mice regardless of the level of supplementation, and the growth curves of mice receiving vitamin A at a level of 1 I.U. per day are similar to those of mice receiving quantities of this vitamin greatly in excess of their needs. These results were duplicated in a second experiment. Hence, vitamin A at a level of one unit per day is as capable of sustaining optimum growth during the period of rapid growth as is vitamin A at levels of 210 to 300 units daily. The weight gains made by

these mice at the various levels of supplementation, summarized in table 3, further substantiate this statement.

b. Mortality rates

Death occurred at approximately the 79th to 85th day in both males and females on the vitamin A-free rations. At the end of 20 months of supplementation, none of the animals

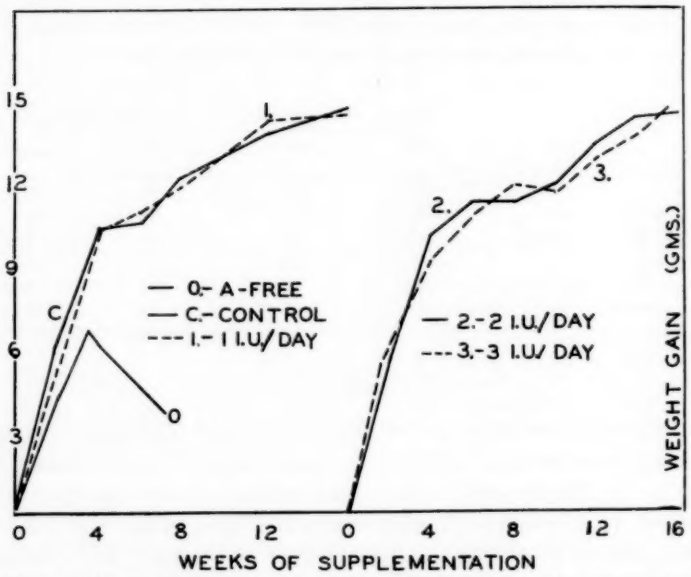

Fig. 1 Average growth rates of male and female mice on the various levels of vitamin A supplementation.

receiving vitamin A had succumbed from natural causes, indicating that vitamin A at a level of 1 I.U. per day is capable of maintaining life for at least this period of time. It was also found that longevity was not beneficially affected by the addition of two, three, or 210 units of vitamin A per day. Among those animals which attained 20 months of age there was an equal distribution of mice from all levels of supplementation.

TABLE 3

Response of mice to the various levels of vitamin A supplementation

GROUP	GAIN IN GRAMS	% WITH OCULAR SYMPTOMS	% DEAD WITHIN 1 YEAR	TOTAL LIVER STORES — I.U. OF VITAMIN A	BLOOD VALUES			REPRODUCTION, % SUCCESSFUL
					RBC (milions/ mm³)	WBC (cells/mm³)	Hb (gm/100 ml)	
				I. Males				
A-free (12)[1]	5.0	100	100					
1 I.U. (12)	23.6	0	0	32.0	7.70	10,950	9.82	100
2 I.U. (12)	23.6	0	0	99.0	7.76	10,586	10.47	100
3 I.U. (12)	24.9	0	0	219.0	7.87	10,770	9.90	100
Control (12)	22.0	0	0	47,100.0	7.76	10,500	11.07	100
				II. Females				
A-free (12)	6.8	100	100					
1 I.U. (12)	21.8	0	0	49.0	9.82	10,400	9.94	
2 I.U. (12)	23.9	0	0	110.0	10.00	10,200	10.75	
3 I.U. (12)	20.7	0	0	218.0	10.31	10,700	10.50	
Control (12)	20.8	0	0	53,000.0	10.07	10,100	10.59	

[1] Numbers in parentheses indicate number of animals in each group.

c. Appearance of the vitamin A deficiency syndrome

Vitamin A at a level of 1 I.U. per day afforded complete protection against the onset of a pathological state due to a deficiency of this vitamin. The first evidence of an avitaminosis appeared in the vitamin A-free mice during an age range of 46 to 64 days. None of the animals receiving vitamin A supplementation showed symptoms of any kind throughout the experimental period. (A majority of the animals were sacrificed for analytical purposes when they were one year old; the remaining mice were sacrificed at 20 months of age.) Post-mortem examinations revealed none of the abnormalities evidenced by mice with a vitamin A deficiency.

d. Reproduction in the male mouse

Since a chronic deficiency of vitamin A in the male mouse results in an almost complete inhibition of reproductory capacity (McCarthy and Cerecedo, '52), it was thought of interest to determine, and to compare, the ability of male mice on varying levels of vitamin A supplementation to exercise this physiological function successfully. Accordingly, males on the one, two, and three unit levels, as well as the positive controls, were mated with virgin females that were kept on stock rations. The data reported in table 3 show that one unit of vitamin A daily was capable of maintaining intact the reproductive function in the male.

e. Liver stores of vitamin A

The ability of the mouse to store vitamin A at the various levels of supplementation was then investigated. It was of special interest to ascertain whether storage of this vitamin could be obtained at a level of 1 I.U. per day. The majority of the animals sacrificed for this purpose were destroyed at approximately one year of age. The few mice sacrificed at an earlier age stored lesser quantities of vitamin A than did the year-old animals. In a few cases no significant storage was found in mice receiving one unit daily when sacrificed

at three, four, or five months of age; others had stored sizeable amounts at the same level of supplementation, when sacrificed at these ages. A tabulation of the amount of vitamin A stored in the livers of mice receiving the various levels of vitamin A supplementation is given in table 3. Vitamin A was determined according to the method of Oser et al. ('43). Whole livers were analyzed individually, since the total amount of vitamin stored was quite low in many cases.

The presence of significant stores of vitamin A in year-old mice receiving 1 I.U. of this vitamin daily would indicate that this level of intake is definitely in excess of the minimum daily requirement of the adult mouse. The fact that some storage occurred even in growing mice would suggest that it is also slightly in excess of the needs of growing animals. Mice receiving 2 I.U. per day stored roughly two to three times the quantity of vitamin A stored by the one-unit mice. The absolute amount stored by the former animals was less than one would expect if the mice utilized only one unit of the administered level. As the intake of the vitamin increases, there is proportionately less economic utilization of the vitamin, as measured by liver storage.

f. Blood values

Since vitamin A seems to be essential for normal cellular construction and function, the effect of the various levels of vitamin A on the blood picture was observed. Koessler et al. ('26) found that on a vitamin A-free diet there was a marked drop in erythrocytes and in hemoglobin in the rat, and that when vitamin A was added to the diet there was a regeneration of hemopoietic function. Abbott and Ahmann ('38) stressed the effect of vitamin A deficiency upon the white blood cell count. When in a state of chronic deficiency, their rats developed a severe anemia. More recently, Fenton, Shrader and Cowgill ('49) noted in mice of the C-57 strain fed a vitamin A-free diet a consistent effect on the blood picture of lowered red blood cell counts, hemoglobin, and hematocrit.

In the present investigation, blood samples were obtained by decapitation. Red blood cell and white blood cell counts, hemoglobin levels, and differential counts were made (table 3). There were no appreciable differences in red blood cell counts and hemoglobin levels among the mice receiving the control diet and the various levels of supplementation. Hence, it would seem that the hemopoietic function in mice receiving 1 I.U. daily is as active as that of mice receiving ample quantities of vitamin A. The white blood cell counts showed considerable variation within each group. Average white blood cell counts for the controls, and for the one- two- and three-unit mice were, respectively, 10,500, 10,955, 10,568, and 10,770 cells/mm^3. The differential counts of the various groups were also comparable. Since there were no marked differences among the differential counts, and since the white blood cell counts were within normal limits, it would seem that 1 I.U. of vitamin A per day is as effective in combating infection as the larger levels administered.

SUMMARY

Albino mice of the Swiss-Webster strain, depleted of tissue stores of vitamin A from birth, received graded doses of vitamin A just prior to the development of a vitamin A deficiency.

It was found that vitamin A at a level of 1 I.U. per day gave as good growth as was obtained at levels of 210 to 300 I.U. daily. This level was also sufficient for the maintenance of life over long periods of time and was adequate in preventing the appearance of internal and external macroscopic symptoms of vitamin A deficiency.

The normal red blood cell counts and hemoglobin levels and the similar white blood cell counts encountered on all levels of vitamin A intake would indicate that 1 I.U. daily supports a normal hemopoietic functioning, and protects against the infections to which chronically vitamin A-deficient mice are susceptible.

The reproductive function in the male is particularly sensitive to a deficiency of vitamin A; 1 I.U. daily allows for a completely normal exercise of this function.

Vitamin A at a level of 1 I.U. per day allows for significant storage in the adult mouse, indicating that this level is definitely in excess of the needs of the adult animal. The fact that storage occurred in mice even during the period of rapid growth would suggest that 1 I.U. daily is somewhat in excess of the needs of the growing animal.

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THE REGISTRY OF NUTRITIONAL PATHOLOGY

The Registry of Nutritional Pathology is a new central agency for the accumulation of material in this specific field. This Registry is a unit of the American Registry of Pathology, within the Armed Forces Institute of Pathology, and is under the general supervision of the Committee on Pathology of the National Research Council. The American Institute of Nutrition, which initiated the project, has the general administrative responsibility and has a Committee to carry out this function.

The registry has 5 main functions:

(1) Accumulation and maintenance of collections of pathologic materials and related case records in special fields of nutritional diseases that are not commonly encountered in individual clinics.
(2) Provide consultation service.
(3) Prepare teaching material.
(4) Establish facilities for the training of students and fellows in the pathology of nutritional diseases.
(5) Facilitate the accessibilty of materials for students and qualified investigators.

Autopsy material and biopsy specimens are solicited. They must be accompanied by a brief clinical history.

In December, 1947, at the suggestion of Dr. Robert E. Johnson and Dr. Robert M. Kark (then, respectively, Scientific Director and Assistant Director of the Army Medical Nutrition Laboratory in Chicago), preliminary work was undertaken at that institution by Dr. Conrad L. Pirani, Chief of the Pathology Division, toward the organization of a Registry of Pathology of Nutritional Diseases. A preliminary draft outlining the purposes, limits and extent of activities of this Registry was prepared at that time.

Every Registry of Pathology must be sponsored by a civilian organization, in most cases a leading national society which is vitally interested in a program and dissemination of knowledge in that particular field of medicine. To this effect, at the Spring meeting, 1948, of the American Institute of Nutrition, Dr. R. E. Johnson proposed that this Society consider sponsoring a Registry of Nutritional Pathology as one unit of the American Registry of Pathology. He pointed out that other established registries (Ophthalmology, Bone Tumors, Gerontology, Urology, Radiology and many others) have been and are valuable for research and teaching in many ways. They have provided collections of material and case records; consultation service for workers in their fields; teaching materials such as microscopic slides, kodachromes and lantern slides in the form of loan collections; training of students and fellows; and they have facilitated accessibility of materials to research workers in a systematic way. In addition, monographs describing and illustrating the areas of the different registries have been published by the Registry Press.

In April, 1949, at its annual meeting, the American Institute of Nutrition assumed the sponsorship of the American Registry of Pathology of Nutritional Diseases. A permanent Committee on the Registry was named at that time, its composition being: Dr. O. A. Bessey, Dr. H. Sebrell, Dr. P. H. Phillips and Dr. R. E. Johnson (Chairman). In subsequent months, organization of the Registry was approved by the National Research Council and by the Surgeon General of the Army.

In January, 1951, Dr. R. E. Johnson resigned from the Chairmanship of the Committee on the Registry of the American Institute of Nutrition and was succeeded a few months later by Dr. Herbert Pollack. Doctor Pollack was able to secure funds [1] for the functioning of the Registry adequate

[1] Grants-in-aid were made by the Milbank Memorial Fund, National Vitamin Foundation and Nutrition Foundation.

for the first few years, and late in 1951 the Registry of Pathology of Nutritional Diseases was activated. Three outstanding pathologists — Dr. P. R. Cannon, Dr. P. Klemperer and Dr. S. B. Wolbach — were named as consultants to the new Registry.

A pamphlet describing in detail the organization and purposes of the Registry of Pathology of Nutritional Diseases, as well as forms to accompany specimens, have been prepared. Both are available on request. Requests and inquiries should be directed to Dr. Herbert Pollack (70 East 77th Street, New York 21, N. Y.) or to Dr. Conrad L. Pirani (808 South Wood Street, Chicago 12, Ill.), respectively, Chairman and Secretary of the Committee on the Registry of the American Institute of Nutrition. Specimens should be sent directly to the Armed Forces Institute of Pathology (7th and Independence, S. W., Washington 25, D. C.).